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$\alpha 5 \beta 1$ -Integrin promotes tension-dependent mammary epithelial cell invasion by engaging the fibronectin synergy site

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ABSTRACT Tumors are fibrotic and characterized by abundant, remodeled, and cross-linked collagen that stiffens the extracellular matrix stroma. The stiffened collagenous stroma fosters malignant transformation of the tissue by increasing tumor cell tension to promote focal adhesion formation and potentiate growth factor receptor signaling through kinase. Importantly, collagen cross-linking requires fibronectin (FN). Fibrotic tumors contain abundant FN, and tumor cells frequently up-regulate the FN receptor $\alpha 5 \beta 1$ integrin. Using transgenic and xenograft models and tunable two- and three-dimensional substrates, we show that FN-bound $\alpha 5 \beta 1$ integrin promotes tension-dependent malignant transformation through engagement of the synergy site that enhances integrin adhesion force. We determined that ligation of the synergy site of FN permits tumor cells to engage a zyxin-stabilized, vinculin-linked scaffold that facilitates nucleation of phosphatidylinositol (3,4,5)-triphosphate at the plasma membrane to enhance phosphoinositide 3-kinase (PI3K)-dependent tumor cell invasion. The data explain why rigid collagen fibrils potentiate PI3K activation to promote malignancy and offer a perspective regarding the consistent up-regulation of $\alpha 5 \beta 1$ integrin and FN in many tumors and their correlation with cancer aggression.

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INTRODUCTION

Tumors are highly fibrotic (Van den Hooff, 1986; Walker, 2001; Lopez-Novoa and Nieto, 2009; Arendt *et al.*, 2010; Ewald *et al.*, 2011; Samuel *et al.*, 2011; Nakagawa *et al.*, 2014). Fibrotic tumors contain abundant quantities of extracellular matrix (ECM) pro-

teins, such as type I collagen, fibronectin (FN), tenascin, and assorted proteoglycans. Cancerous tissues also exhibit altered levels and activity of ECM receptors, including integrins, discoidin receptors, and syndecans (Ioachim *et al.*, 2002; West *et al.*, 2005;

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Abbreviations used: 2D, two-dimensional; 3D, three-dimensional; ANOVA, analysis of variance; BAPN, β -aminopropionitrile; BSA, bovine serum albumin; ECM, extracellular matrix; eGFP, enhanced green fluorescent protein; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FITC, fluorescein isothiocyanate; FN, fibronectin; FRAP, fluorescence recovery after photobleaching; FRET, fluorescent resonance energy transfer; HRP, horseradish

peroxidase; IgG, immunoglobulin G; LOX, lysyl oxidase; mAb, monoclonal antibodies; MEC, mammary epithelial cell; MyPT, myosin phosphatase binding protein; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PHSRN, proline-histidine-serine-arginine-asparagine; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol (3,4)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate; rBM, reconstituted basement membrane; Tet, tetracycline; TIRF, total internal reflection fluorescence; VEGF, vascular endothelial growth factor.

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Desgrosellier and Cheresch, 2010; Nam et al., 2010; Ozbek et al., 2010; Gehler et al., 2013; Pickup et al., 2014; Theocharis et al., 2015). Elevated $\beta 1$ -integrin levels and focal adhesion kinase (FAK) activity correlate positively with high tumor grade and predict for poor patient prognosis (Recher et al., 2004; dos Santos et al., 2012; Sulzmaier et al., 2014). Inhibiting $\beta 1$ -integrin ligand binding represses the malignant phenotype of tumor cells in vitro and in vivo (Weaver et al., 1997; Stoeltzing et al., 2003; Huang et al., 2011; Blandin et al., 2015), and transgenic ablation of $\beta 1$ integrin or FAK prevent oncogene-induced malignant transformation and metastasis (van Nimwegen et al., 2005). Function-blocking antibodies to integrins and FAK inhibitors are also under clinical consideration to improve the treatment of fibrotic tumors (Walsh et al., 2010; Stokes et al., 2011; Lagares et al., 2012; Jiang et al., 2016). These findings emphasize the interplay among tissue fibrosis, the ECM, and integrin signaling in malignancy.

Tumors are mechanically corrupted tissues with high interstitial pressure, elevated compression, increased solid stress, ECM stiffening, and increased cellular tension (Heldin et al., 2004; Paszek et al., 2005; Levental et al., 2009; Lopez et al., 2011; Samuel et al., 2011; Provenzano et al., 2012; Mouw et al., 2014; Laklai et al., 2016). Fibrotic tumors contain a stiffened ECM, which associates with cancer aggression and high patient mortality (Shimosato et al., 1980; Maeshima et al., 2002; Willis and Borok, 2007; Levental et al., 2009; Egeblad et al., 2010). Elevating tumor cell contractility (tension) or stiffening the tumor ECM through increased deposition, remodeling, and cross-linking of type 1 fibrillar collagen promotes the malignant transformation and aggressiveness of a tumor tissue in culture and in vivo (Paszek et al., 2005; Levental et al., 2009; Kraning-Rush et al., 2012; Acerbi et al., 2015; Laklai et al., 2016). Moreover, reducing cell tension or interstitial pressure or preventing matrix stiffening by inhibiting collagen cross-linking decreases tumor incidence and aggression and can improve treatment efficacy (Paszek et al., 2005; Levental et al., 2009; Samuel et al., 2011; Provenzano et al., 2012; Chauhan et al., 2013; Miller et al., 2015). Cell tension and ECM stiffness promote focal adhesion assembly, and $\beta 1$ -integrin ligation and signaling is necessary for tension-dependent malignant transformation and tumor metastasis (Paszek et al., 2005; Levental et al., 2009; Samuel et al., 2011; Pickup et al., 2013; Kaushik et al., 2016). What remains unclear is whether tissue mechanics promote malignancy and tumor aggression by engaging specific $\beta 1$ -integrin heterodimers, and if so, why? Indeed, while thick perpendicular collagen fibers associate with invasive cancers (Provenzano et al., 2006; Levental et al., 2009; Conklin et al., 2011; Carey et al., 2012), and a stiffened collagen ECM can promote malignant transformation in culture (Paszek and Weaver, 2004; Levental et al., 2009; Miroshnikova et al., 2011; Cassereau et al., 2015), the major collagen receptor, $\alpha 2\beta 1$ integrin, inhibits rather than promotes malignancy in vivo (Zutter et al., 1995; Ramirez et al., 2011).

FN is frequently elevated in primary and metastatic tumors (Nam et al., 2010), and the premetastatic niche contains abundant FN (Kaplan et al., 2005). Moreover, expression of the major FN receptor $\alpha 5\beta 1$ integrin is increased in many solid primary tumors, and the levels of either FN or $\alpha 5\beta 1$ integrin independently correlate with poor prognosis in cancer patients (Yao et al., 2007; Dingemans et al., 2010; Nam et al., 2010). Functionally, ligated $\alpha 5\beta 1$ integrin promotes tumor cell growth and survival, and blocking FN ligation by soluble tripeptide composed of L-arginine, glycine, and L-aspartic acid (RGD) sensitizes breast tumor spheroids to radiation treatment (Park et al., 2006; Nam et al., 2010; Yao et al., 2016). Importantly, FN can be coassembled with type I collagen (Sevilla et al., 2013), and lysyl oxidase (LOX)-dependent collagen cross-linking requires FN

(Kubow et al., 2009; Cox et al., 2013). The binding of FN to cross-linked collagen increases the ability of fibroblasts to unfold FN to reveal cryptic binding sites, including the synergy site (Friedland et al., 2009; Seong et al., 2013), which is uniquely ligated by $\alpha 5\beta 1$ integrin (Garcia et al., 2002). Cells with FN-synergy site-bound $\alpha 5\beta 1$ integrin engage an adhesive bond that enables them to exert higher forces on their ECM (Friedland et al., 2009; Kong et al., 2009; Benito-Jardon et al., 2017). Consistently, tumor cells sorted for higher cell-surface $\alpha 5\beta 1$ integrin are more contractile and migrate faster on a rigid FN ECM (Mierke et al., 2011). This suggests that a stiff, collagen-FN-rich ECM may favor ligation of the FN synergy site by $\alpha 5\beta 1$ integrin to enhance integrin-growth factor receptor signaling that fosters malignancy. Here we used mouse models of mammary cancer, three-dimensional (3D) organotypic mammary epithelial cell (MEC) cultures, and a force reactor with quantitative analysis to explore the relationship between a stiff collagen ECM, FN ligation of the synergy site by $\alpha 5\beta 1$ integrin, and tumor cell invasion.

RESULTS

Mammary tumor invasion colocalizes with collagen and FN fibers that decrease when stromal stiffening and malignancy are inhibited

We first determined whether an association exists between FN and ECM collagen stiffness-dependent mammary tumor invasion in vivo. We looked for colocalization of collagen and FN and tumor cell invasion in the mammary glands of late-stage FVB PyMT tumors that atomic force microscopy indentation previously revealed was significantly stiffer than that of a normal mammary gland (normal gland: 0.4 kPa, PyMT tumor: 3 kPa) (Lopez et al., 2011; Pickup et al., 2013). Confocal images of PyMT mammary tumor tissue immunostained for FN (Figure 1A, green) and imaged by second harmonic generation (Figure 1A, light blue) showed that a high percentage of invading tumor cells (red) could be observed on thick collagen fibers that costained with FN (Figure 1A, quantified in Figure 1B). To implicate FN in tension-dependent tumor invasion, we next analyzed mammary tissue from MMTV-Neu mice treated with (LOX-i) and without (control) the small-molecule inhibitor β -aminopropionitrile (BAPN) that inhibits LOX activity and that we previously showed prevents collagen cross-linking and ECM stiffening and reduces tumor progression (Siegel, 1974; Kagan, 2000; Kirschmann et al., 2002; Levental et al., 2009). Confocal imaging of mammary tumors from the BAPN-treated mice confirmed that the tumors had lower cell tension compared with their nontreated controls, as revealed by decreased immunostaining for myosin activity (Figure 1C, "pMLC," green, quantified in Figure 1D), reflecting the decreased ECM stiffness of the tissue (PyMT tumor: 3 kPa; PyMT tumor + BAPN: 0.1 kPa) (Levental et al., 2009; Lopez et al., 2011). Immunofluorescence imaging analysis further indicated that the BAPN-inhibited tumors had reduced integrin mechanosignaling (Dumbauld et al., 2010), as indicated by lower levels of FAK phosphorylation at residue 397 (Figure 1C, "p³⁹⁷FAK," red, quantified in Figure 1D). Intriguingly, immunostaining showed that the BAPN-treated mice also had lower $\alpha 2$ integrin (Figure 1C, "Integrin $\alpha 2$," red) and $\alpha 5$ integrin (Figure 1C, "Integrin $\alpha 5$," red), as well as reduced tissue FN (Figure 1C, "FN," green, quantified in Figure 1D). These findings provide evidence of a plausible relationship among FN, collagen, and stiffness-dependent tumor cell invasion.

A link between the malignant phenotype of mammary tumors and expression of FN and its integrin receptors

To explore the possibility of a relationship between FN and the malignant phenotype of MECs, we conducted reconstituted basement

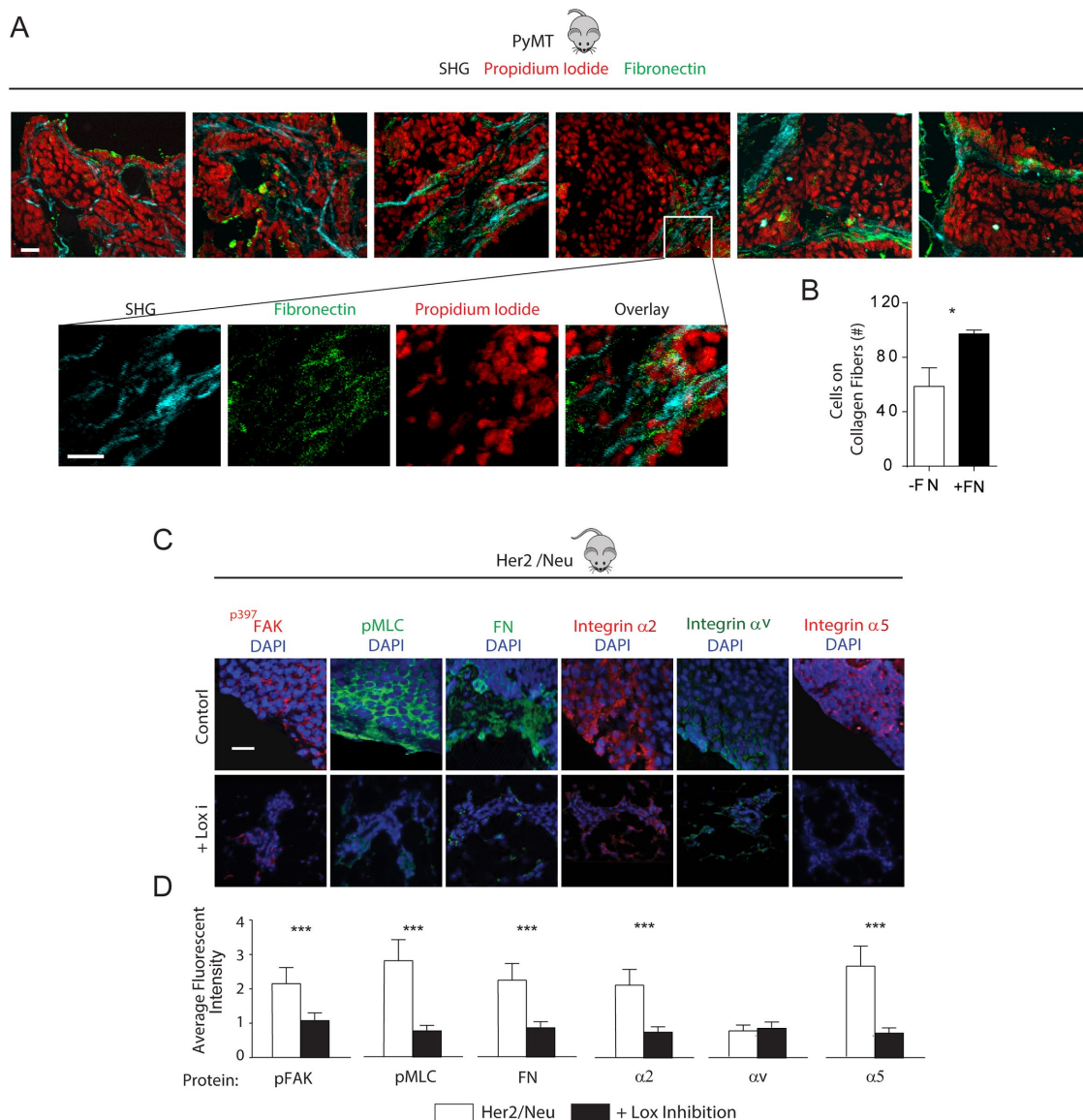


FIGURE 1: Mammary tumor invasion colocalizes with collagen and FN fibers that decrease when stromal stiffening and malignancy are inhibited. (A) Zoomed-in second harmonic generation images of collagen (blue), FN (green), and nuclei (red), as well as an overlay in the 7-mo-old PyMT tumors at the invasive front (left four images) evident from the zoomed-out overlay image (rightmost image). Scale bar: 20 μ m. (B) Quantification of cells migrating along FN-coated vs. non-FN-coated collagen fibers. (C) Confocal immunofluorescence images of mammary tissue stained for p³⁹⁷FAK, pMLC, FN, and α 2 integrin, α v integrin, and α 5 integrin in tissue excised from 7-mo-old control (Her2/Neu) or LOX inhibitor-treated (+LOX-i) Her2/Neu transgenic mice with quantification of fluorescence intensity in D. Scale bar: 10 μ m. Results are the mean \pm SEM of three separate experiments. For animal studies, three animals were used per condition with three to five images per animal analyzed for quantification. *, $p < 0.05$; ***, $p < 0.001$.

membrane (rBM) mammosphere assays using the immortalized MEC tumor progression series HMT3522 (Weaver *et al.*, 1996, 1997). As previously reported, the nonmalignant S1 MECs in this tumor-progression series formed growth-arrested polarized acini when grown within a 3D rBM for 2 wk (Weaver *et al.*, 1997; Wang *et al.*, 1998; Rizki *et al.*, 2008). The polarized acini phenotype was indicated by apical-lateral cortical actin (phalloidin, green, Figure 2A), cell-cell-localized β -catenin (β -catenin, green, Figure 2A, left) and basally localized β 4 integrin (Figure 2A, left). The S1 MECs also assembled an endogenous basement membrane, as indicated by basally deposited laminin 5 (Laminin V) (Figure 2A, left). By comparison, the malignant T4-2 MECs formed continuously growing (unpublished data), tumor cell

colonies, lacking apical-basal polarity and stable cell-cell adhesions (Figure 2A, middle). Flow-activated cell sorting (FACS) revealed that the HMT-3522 T4-2 cells in this series express significantly higher plasma membrane levels of the FN integrin receptors α v and α 5 and β 1 (Figure 2B), whose levels were confirmed by immunoblot analysis of protein isolated from 3D rBM colonies (Figure 2C). Organotypic rBM cultures also revealed that only the tumor colonies deposited FN in the 3D rBM assay (Figure 2A, middle, "Fibronectin"). Importantly, inhibiting β 1-integrin ligand binding, using the ligand function-blocking antibody (A1B2; Weaver *et al.*, 1997) or by pharmacologically inhibiting the activity of the epidermal growth factor receptor (EGFR) by treating the cultures with tyrphostin (Wang *et al.*, 1998),

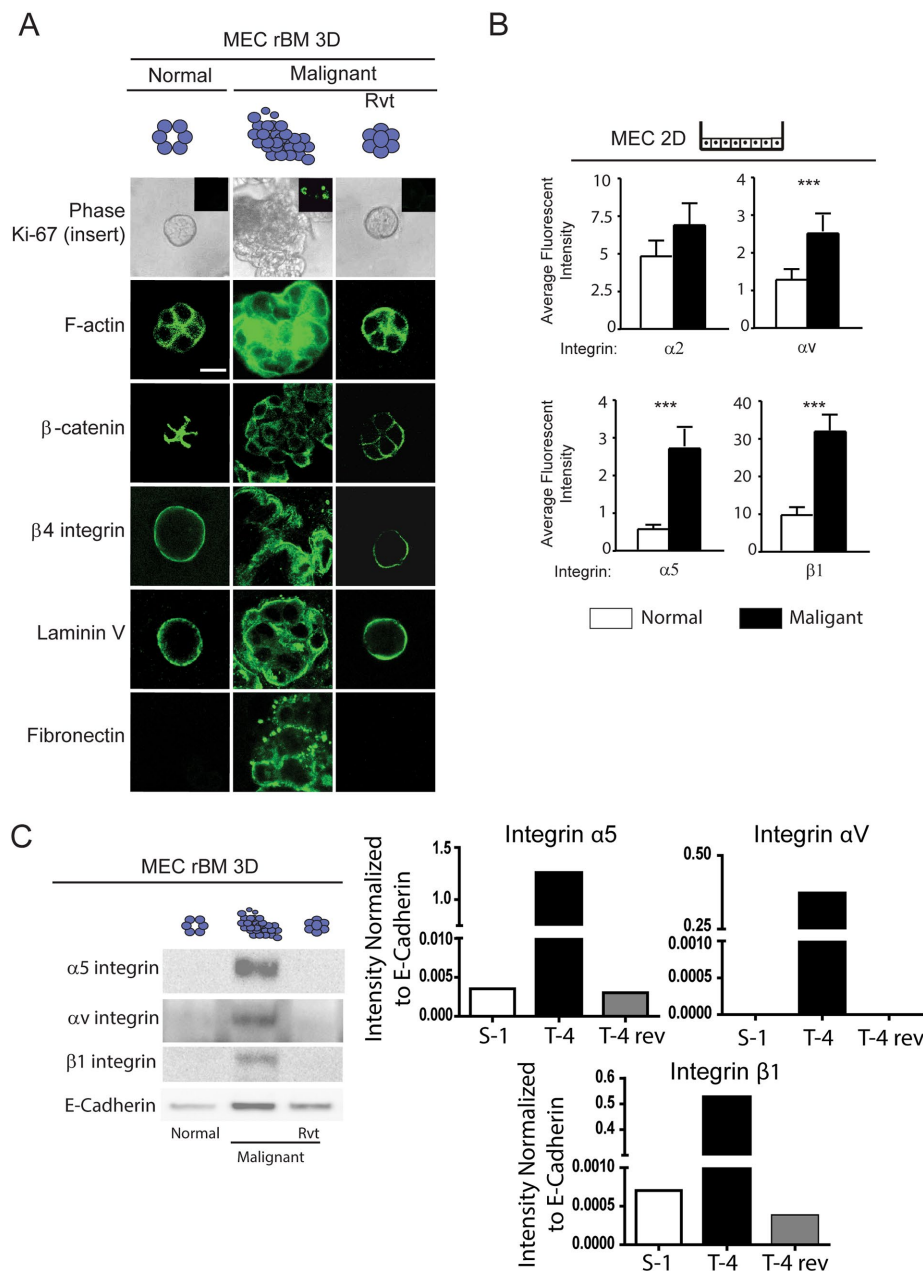


FIGURE 2: Link between the malignant phenotype of mammary tumors and expression of FN and its integrin receptors. (A) Phase-contrast and confocal immunofluorescence images of Ki-67 (insert), phalloidin (F-actin), β -catenin, $\beta 4$ integrin, laminin-5, and FN-stained colonies of nonmalignant (S-1), malignant (T4-2), and phenotypically reverted (T4 Rvt) HMT-3522 human MECs grown within an rBM for 2 wk. Scale bar: 10 μ m. (B) Bar graphs of FACS analysis of membrane-localized integrins in S-1 compared with T4-2 MECs. (C) Representative immunoblot image of $\alpha 5$, αv , and $\beta 1$ integrin in lysates from S-1, T4-2, and T4 Rvt 3D rBM colonies shown in A with corresponding quantification of signal intensity normalized to E-cadherin loading control. Fifty acini were analyzed in three separate experiments. ***, $p < 0.001$.

reverted the invasive colony phenotype of the T4-2 MECs toward that of the differentiated nonmalignant S1 MECs (Weaver *et al.*, 1997; Weigelt *et al.*, 2014). Thus the reverted T4-2 MECs formed growth-arrested (unpublished data), apically-basally polarized acini after 2 wk of growth within a 3D rBM (Figure 2A, right). Confocal images of immunofluorescently stained cultures showed that the reverted tumor colonies had apical-lateral cortical actin (Figure 2A, right, "F-actin," green) and cell-cell-localized β -catenin (Figure 2A, right, " β -catenin," green). Immunostaining also revealed that the

reverted tumor colonies had basally localized $\beta 4$ integrin (Figure 2A, right, " $\beta 4$ integrin," green) and assembled an endogenous basement membrane, as revealed by basally deposited laminin 5 (Figure 2A, right, "Laminin V," green). Interestingly, the reverted tumor colonies no longer secreted FN into the 3D rBM (Figure 2A, right, "Fibronectin," green) and an immunoblot of total colony protein showed a dramatic reduction of integrin $\alpha 5$, $\alpha 2$, and $\beta 1$ in the reverted acini (Figure 2C). These findings establish a causal association between FN and its integrin receptors $\alpha 5$, αv , and $\beta 1$ and expression of the malignant phenotype of mammary tissue-like structures in 3D rBM culture.

FN-ligated $\alpha 5 \beta 1$ integrin regulates the malignant phenotype of MECs in vitro and in vivo

We next asked whether FN ligation by either of its integrin receptors, $\alpha 5$ or αv , influenced the malignant phenotype of MEC HMT-3522 tumor cells. We treated the HMT-3522 T4-2 MECs, which secrete FN (see Figure 2A), with either $\alpha 5$ - or αv -integrin function-blocking antibodies and assayed for changes in cell growth and colony morphology after 2 wk within rBM compared with the effect of blocking the activity of $\alpha 2$, $\alpha 3$, or $\beta 1$ integrin. Blocking ligand binding to αv (Figure 3A, second column), $\alpha 2$ (Figure 3A, third column), or $\alpha 3$ integrin (unpublished data) had little to no effect on the growth and morphological behavior of the T4-2 MECs in a 3D rBM. However, inhibiting $\alpha 5$ integrin repressed the malignant phenotype of the T4-2 MECs in rBM (Figure 3A, fourth column), similar to what was observed following inhibition of $\beta 1$ -integrin ligand binding (Figure 3A, fifth column; see also Figure 2A). After 2 wk of culture within rBM the T4-2 MECs treated with either immunoglobulin G (IgG) isotype-matched control or inhibitory antibodies to $\alpha 2$ or αv integrin formed continuously growing, large, disorganized, and invasive colonies in rBM, as indicated by disorganized β -catenin (Figure 3A, top), $\alpha 6$ integrin (Figure 3A, middle), and actin (Actin; Figure 3A, bottom). By contrast, the T4-2 MECs treated with function-blocking antibodies against either $\alpha 5$ or $\beta 1$ integrin assembled morphologically reverted colonies in rBM that were reminiscent of differentiated nonmalignant S1 mammary acini (Figure 2A). T4-2 MECs in which $\alpha 5 \beta 1$ integrins were inhibited assembled growth-arrested (unpublished data), polarized structures as indicated by cell-cell-localized β -catenin (Figure 3A; top panel), basal $\alpha 6$ integrin (Figure 3A, middle), and apical-lateral cortical actin (Figure 3A, bottom). The reverted T4-2 MECs also formed acini that were at least 60–70% smaller than the nonreverted colonies (Figure 3B). Moreover, preventing $\alpha 5$ - or $\beta 1$ -integrin ligand binding significantly impaired

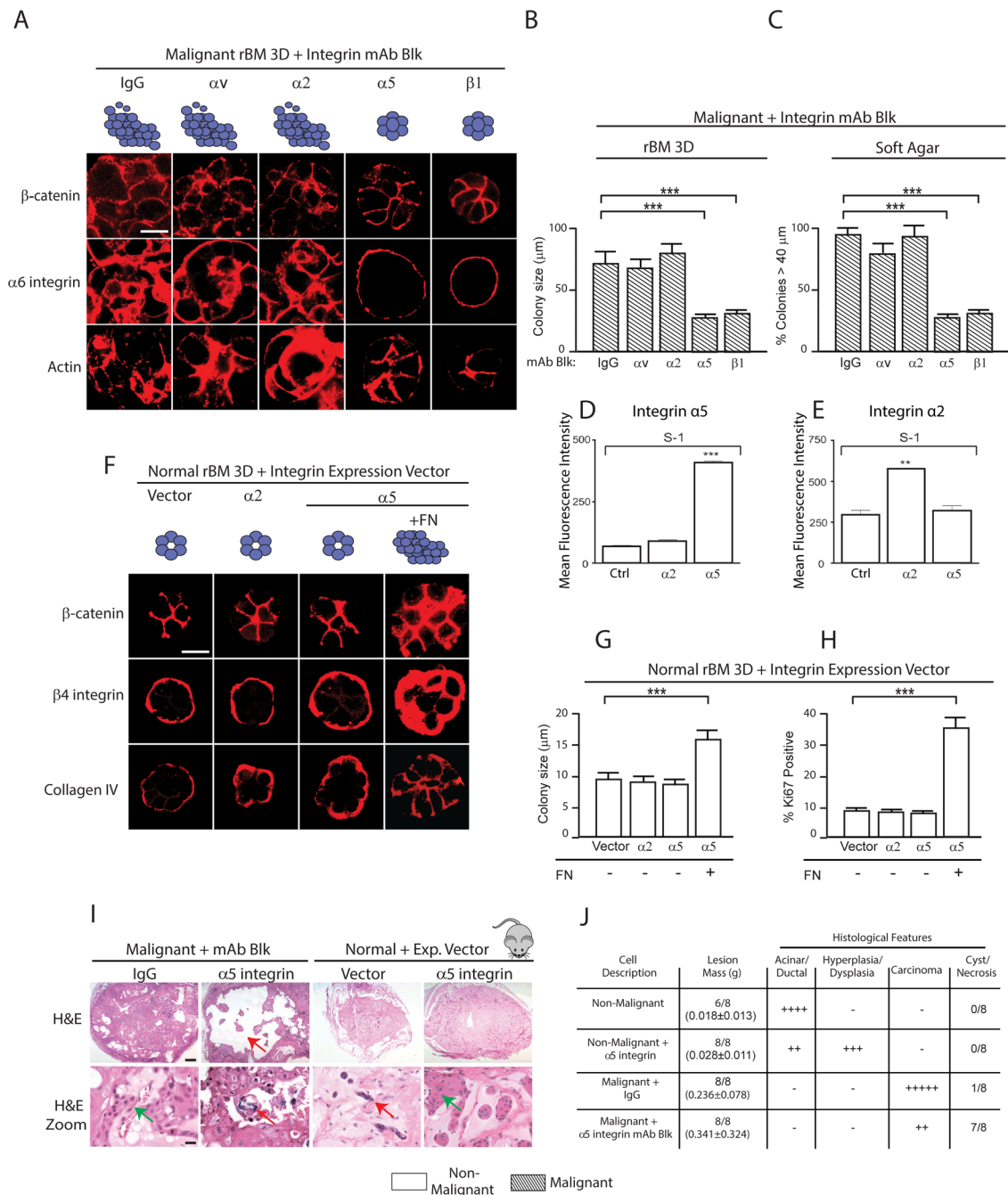


FIGURE 3: FN-ligated $\alpha 5 \beta 1$ integrin regulates the malignant phenotype of MECs in vitro and in vivo. (A) Confocal immunofluorescence images of β -catenin, $\alpha 6$ integrin, and actin (Phalloidin) staining of malignant (T4-2) MEC colonies grown for 2 wk in rBM in the presence of a function-blocking antibody (mAb) to αv , $\alpha 2$, $\alpha 5$, or $\beta 1$ or an IgG isotype-matched control mAb. Scale bar: 30 μm . (B) Bar graph showing relative size of the T4-2 colonies shown in A. (C) Bar graph showing percentage of tumor colonies formed in soft agar (40+ microns) following treatment with function-blocking mAbs to αv , $\alpha 2$, $\alpha 5$, or $\beta 1$ integrin or an IgG isotype-matched control mAbs. (D) Mean fluorescence intensity of integrin $\alpha 2$ and $\alpha 5$ expression in S-1 cells overexpressing *Itga2*. (E) Mean fluorescence intensity of integrin $\alpha 2$ and $\alpha 5$ expression in S-1 cells overexpressing *Itga5*. (F) Confocal immunofluorescence images of β -catenin, $\beta 4$ integrin, and collagen IV staining of colonies of nonmalignant (S-1) vector (Ctrl) MECs and MECs expressing elevated $\alpha 2$ or $\alpha 5$ integrin grown in rBM with or without the addition of FN (+FN) for 2 wk. Scale bar: 10 μm . (G) Bar graph showing relative size of S-1 MEC colonies shown in D. (H) Bar graph showing percent Ki-67-positive S-1 MEC colonies shown in D. (I) Bright-field images of low (top) and high (bottom) magnification of H&E sections of tissue excised 2 mo following injection of malignant T4-2 MECs with IgG or a function-blocking antibody to $\alpha 5$ integrin and nonmalignant S-1 MECs expressing empty vector or an $\alpha 5$ integrin. Scale bar: 10 μm . Red arrows indicate areas of necrosis observed upon blocking of integrin $\alpha 5$. Red arrows indicate hyperplastic or dysplastic cellular structures. (J) Table summarizing tumor score and histological features. Scale bar: 10 μm . **, $p < 0.01$; ***, $p < 0.001$.

the anchorage-independent growth and survival of the T4-2 MECs in soft agar (Figure 3C). These data suggest that FN-ligated $\alpha 5 \beta 1$ integrin regulates expression of the malignant phenotype of the T4-2 MEC in a 3D rBM.

We next investigated whether FN-ligated $\alpha 5 \beta 1$ integrin could promote the malignant phenotype of nonmalignant MECs. HMT-3522 S1 nonmalignant MECs (which express negligible $\alpha 5$ integrin) were engineered to express either a tetracycline (Tet)-regulated enhanced green fluorescent protein (eGFP)-tagged $\alpha 5$ integrin or an eGFP-tagged $\alpha 2$ integrin. Immunoblot and FACS analysis confirmed a Tet-modulated increase in total and cell-surface $\alpha 5$ integrin and $\alpha 2$ integrin in two independent nonmalignant HMT-3522 S1 MEC lines (Figure 3, D and E) and two independent nonmalignant MCF10A MEC lines (Damiano *et al.*, 2014). Neither ectopic expression of $\alpha 5$ integrin nor $\alpha 2$ integrin significantly altered plasma membrane levels of $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 1$, or $\beta 4$ integrin in either the HMT-3522 S1 or the MCF10A MECs (unpublished data). Furthermore, MCF10A MECs expressing elevated levels of $\alpha 5$ integrin showed greater adhesion to FN, and those engineered to express high $\alpha 2$ integrin adhered more to a type I collagen ECM (Damiano *et al.*, 2014). The nonmalignant S1 MECs expressing either more $\alpha 2$ integrin or $\alpha 5$ integrin assembled growth-arrested (Figure 3F), polarized acini with a cleared central lumen after 10–14 d of growth within rBM. This “normal” phenotype was indicated by immunofluorescence staining, which revealed cell–cell-localized β -catenin (Figure 3F, top), basally localized $\beta 4$ integrin (Figure 3F, middle), and basally deposited collagen IV (Figure 3F, bottom). Importantly, when the $\alpha 5$ integrin was ligated through addition of FN to the rBM, the HMT-3522 S1 MECs expressing ectopic $\alpha 5$ integrin formed continuously growing colonies (Figure 3, G and H) that by 2 wk were almost double the size of the colonies formed by the non-FN-ligated $\alpha 5$ integrin-expressing S1 MECs (Figure 3G). The FN-ligated $\alpha 5$ integrin-expressing S1 MEC colonies were unable to clear their lumens (Figure 3F), showed diffuse cell–cell-localized β -catenin (Figure 3D, top) and disorganized $\beta 4$ integrin (Figure 3F, middle), and failed to assemble an endogenous collagen IV basement membrane (Figure 3F, bottom). In the presence of exogenous FN, nonmalignant S1 MECs expressing elevated cell-surface $\alpha 5$ integrin formed colonies that were 30–40% larger than the S1 MECs expressing higher $\alpha 2$ integrin, despite the availability of abundant collagen (Figure 3G). We observed a similar tumor-promoting phenotype following FN ligation in the nonmalignant immortalized MCF10A MECs ectopically engineered to express higher $\alpha 5$ -integrin levels (Figure 4A) (Damiano *et al.*, 2014) with little to no effect on tissue phenotype following collagen ligation of MCF10A MECs expressing higher $\alpha 2$ -integrin levels (Figure 4A). These findings demonstrate that FN-ligated $\alpha 5 \beta 1$ integrin can promote the malignant phenotype of nontransformed MEC tissue-like structures in vitro, reminiscent of prior studies implicating FN ligation on acinar morphogenesis and growth of MCF10A (Williams *et al.*, 2008).

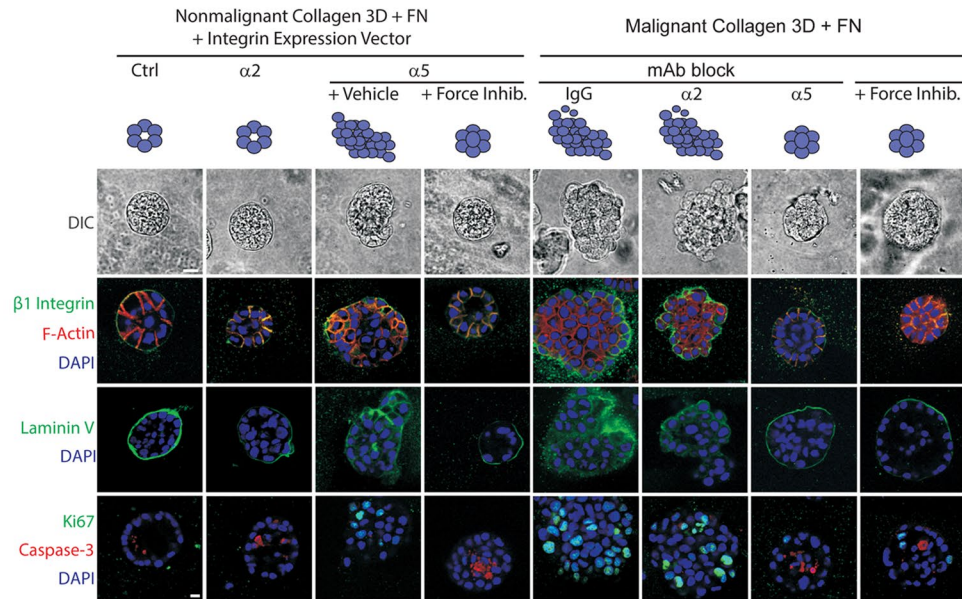
To further implicate FN ligation of $\alpha 5 \beta 1$ integrin in the malignant behavior of mammary tissues, we manipulated $\alpha 5$ -integrin expression and function in nonmalignant and malignant MECs and assayed for effects on their malignant behavior in vivo. We inoculated nonmalignant HMT-3522 S1 MECs expressing eGFP (nonmalignant control) or $\alpha 5$ integrin (+ $\alpha 5$ integrin), and HMT-3522 T4-2 malignant MECs treated with either an $\alpha 5$ -integrin function-blocking monoclonal antibody ($\alpha 5$ -integrin inhibited) or an isotype IgG control antibody (tumor control), into the rear flanks of BALB/c nu/nu mice. Two months following MEC inoculation, the mice were killed and the tumor phenotype was assessed. The control tumors had formed large, actively growing, invasive, and angiogenic tumor masses, as indicated by

elevated proliferating cell nuclear antigen (PCNA) immunostaining, negligible activated caspase 3 immunostaining, a clearly visible vasculature (confirmed by CD34 immunostaining), and histopathological analysis that confirmed the presence of invasive cell masses (Figure 3, I and J, green arrows indicate proliferating cells, and Supplemental Figure 1C, left panels). By comparison, T4-2 cells treated with the $\alpha 5$ -integrin function-blocking antibody formed only small, nonproliferating tumor colonies that immunostained positively for the apoptosis marker-activated caspase 3. The tumors formed by the cells treated with the $\alpha 5$ -integrin function-blocking antibody also lacked a vasculature (Supplemental Figure 1D, top left panels), as revealed by negligible CD34 immunostaining (Supplemental Figure 1D, bottom left panels), and they showed histopathological evidence of cystic degeneration and necrosis (Figure 3, I and J, red arrows indicate dying cells, and Supplemental Figure 1C, right panels). As expected, the majority of the injected nonmalignant S1 MECs failed to survive (red arrow indicate dying cells), and those that did formed ductal-like differentiated tissue-like structures (Figure 3I, zoom, see arrows; Figure 3J). By contrast, those S1 MECs expressing high levels of $\alpha 5$ integrin not only survived, but they grew to form hyperplastic/dysplastic cell masses that apparently induced an angiogenic response, as indicated by a visible vasculature and positive CD34 tissue immunostaining (Figure 3, I and J, green arrows point to proliferating cells, and Supplemental Figure 1D, top and bottom right panels). These in vivo tumor studies suggested that engagement of $\alpha 5 \beta 1$ integrin by MECs might stimulate tumor angiogenesis, another key feature attributed to a malignant tissue (Pickup *et al.*, 2014). To directly address this possibility, we grew the nonmalignant S-1 MECs ectopically expressing $\alpha 5$ integrin or $\alpha 2$ integrin within a 3D rBM doped with either type I collagen or FN inside of a tissue culture insert until they formed colonies (10–12 d culture). The tissue culture inserts containing the rBM/FN-embedded MEC colonies were then transferred into a Transwell in which vascular endothelial cells were grown on the bottom to semiconfluence and then overlaid with a layer of type I collagen (Supplemental Figure 1A). The growth and invasion of the vascular endothelial cells into the collagen gel was then monitored over several days, and extent of network formation was quantified (60 d). Consistent with the in vivo findings, we noted that the colonies formed by the nonmalignant MECs expressing FN-ligated $\alpha 5$ integrin, but not collagen-ligated $\alpha 2$ integrin, induced more endothelial network-like structures (Supplemental Figure 1B, top left bar graphs). We next conducted a similar endothelial network assay using the malignant HMT-3522 T4-2 MECs incubated with IgG control antibody or function-blocking antibodies against $\alpha 2$, αv , $\alpha 5$, or $\beta 1$ integrin. Findings showed that only the T4-2 colonies incubated with the IgG control or the $\alpha 2$ - or αv -integrin function-blocking antibodies were able to stimulate a significant amount of vascular network formation (Supplemental Figure 1B) compared with those tumor cells that had been phenotypically reverted by blocking either $\alpha 5$ - or $\beta 1$ -integrin function (Supplemental Figure 1B, top right bar graphs). Consistent with these findings, enzyme-linked immunosorbent assay (ELISA) revealed that only the FN-ligated $\alpha 5 \beta 1$ -integrin MECs expressed abundant vascular endothelial growth factor (VEGF), and that inhibiting $\alpha 5$ or $\beta 1$ integrin in the malignant T4-2 MECs significantly reduced levels of tumor-secreted VEGF (Supplemental Figure 1B, bottom right and left bar graphs). These findings indicate that FN-ligated $\alpha 5 \beta 1$ integrin can regulate the malignant phenotype of mammary tissues both in vitro and in vivo.

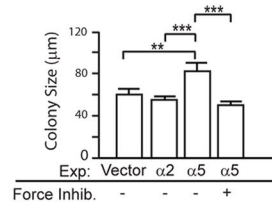
FN-ligated $\alpha 5 \beta 1$ integrin increases cell tension to promote mammary malignancy

We next determined whether the ability of FN ligation of $\alpha 5 \beta 1$ integrin to promote the malignant behavior of mammary tissues

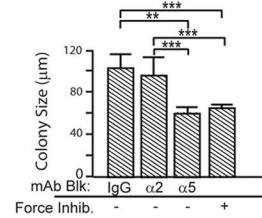
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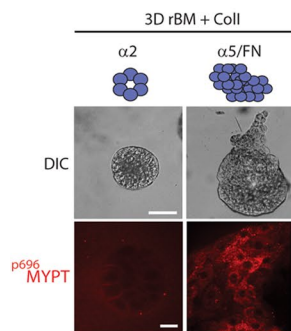
B Collagen 3D + FN + Integrin Expression Vector



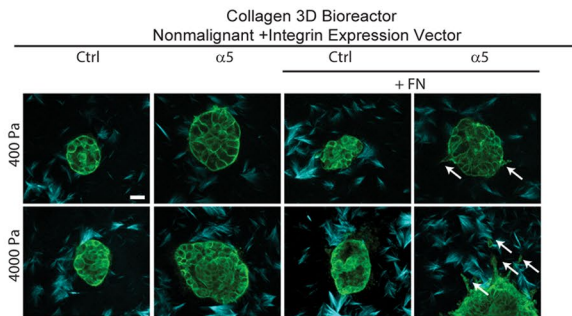
C Collagen 3D + FN



D



E



F

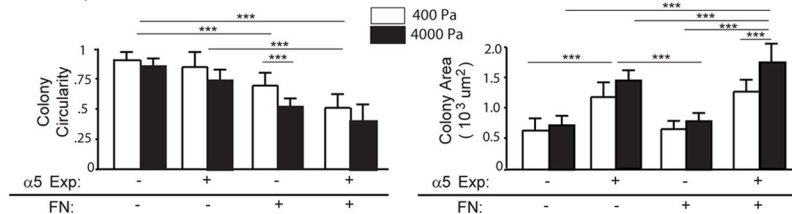


FIGURE 4: FN-ligated $\alpha 5 \beta 1$ integrin increases cell tension to promote mammary malignancy. (A) Phase-contrast and confocal immunofluorescence images of $\beta 1$ integrin, phalloidin (F-actin), laminin V, Ki-67 and activated caspase 3 (Caspase-3), and DAPI (nuclei)-stained colonies of nonmalignant (MCF10A) human MECs expressing empty vector or elevated $\alpha 2$ or $\alpha 5$ integrin treated with or without a ROCK inhibitor and malignant cells (HMT-3522 T4-2) incubated with nonspecific IgG or function-blocking antibodies to $\alpha 2$ or $\alpha 5$ integrin or treated with or without a ROCK inhibitor grown within a collagen gel with added FN for 2 wk. Scale bar: 8 μ m. (B) Bar graph showing relative size of the nonmalignant colonies shown in A. (C) Bar graph showing relative size of the malignant colonies shown in A. (D) Immunofluorescence confocal images (bottom) and phase-contrast images (top) of 3D cultures of $\alpha 2$ /Col 1 and $\alpha 5$ /FN nonmalignant MECs stained for the ROCK target p696MYPT (top). Scale bar: 50 μ m. (E) Second harmonic generation images of nonmalignant (MCF10A) human mammary epithelial day 20 acini (green) expressing empty vector or elevated $\alpha 5$ integrin with or without FN embedded in collagen (blue) and installed into a 3D tension bioreactor system and subjected either to 0% (400 Pa) or 10% (4000 Pa) stretch, as described previously (Cassereau *et al.*, 2015). Arrows in E indicate invasive cells and colony circularity (left) and area (right) are quantified in F. Results are the mean \pm SEM of three to five separate experiments. **, $p < 0.01$; ***, $p < 0.001$. (Ten to 15 acini were analyzed per condition in three separate experiments.)

depended on cell tension. We treated 3D collagen–FN cultures of the nonmalignant MCF10A MECs engineered to express higher cell-surface $\alpha 5$ integrin or $\alpha 2$ integrin, with either a ROCK inhibitor (Y27632, 10 μ M) (Figure 4A) or a myosin inhibitor (blebbistatin; unpublished data). Two weeks following embedment within a 3D collagen–rBM–FN hydrogel, vector control, nonmalignant MCF10A MECs, and the MCF10A MECs ectopically expressing higher $\alpha 2$ integrin assembled growth-arrested (Figure 4A, bottom, “Ki-67”), tissue-like structures (Figure 4A, top, DIC phase-contrast images) that resembled acini. Confocal immunofluorescence images confirmed that both the MCF10A vector control, and $\alpha 2$ integrin–overexpressing MEC acini were polarized. Colony phenotype was indicated by apical-lateral cortical actin (Figure 4A, second row, “ $\beta 1$ integrin,” “F-actin,” and “DAPI”); deposition of an endogenous basally localized, basement membrane (Figure 4A, third row, “Laminin V”); and lumen clearance, indicated by detectable apoptotic cells in the colony core (Figure 4A, bottom row, activated “Caspase-3” and “DAPI”). By contrast, the MCF10A MECs ectopically expressing higher $\alpha 5$ integrin formed continuously growing, disorganized colonies lacking a central lumen (Figure 4A, third column) that were significantly larger than those formed by either the vector control or $\alpha 2$ integrin–overexpressing MECs (Figure 4B). Critically, treatment of the MCF10A MEC $\alpha 5$ integrin–overexpressing MEC colonies with ROCK inhibitor phenotypically reverted the colonies so they resembled growth-arrested, differentiated acini, with a size and organization that resembled the vector control MCF10A MEC acini (Figure 4A, compare fourth to first column, and Figure 4B). These findings suggest FN ligation of $\alpha 5\beta 1$ integrin promotes a tumor phenotype in MECs by increasing cell tension. Consistent with this notion, immunostaining revealed that the FN-ligated MECs expressing higher $\alpha 5$ integrin, but not the collagen-ligated MECs expressing higher $\alpha 2$ integrin, stained positively for activity of the ROCK target, myosin phosphatase binding protein (MyPT) (p^{696} MyPT) (Figure 4D). Similarly, treating the malignant HMT-3522 T4-2 MECs that overexpressed $\alpha 5\beta 1$ integrin (Figure 2), which we previously showed had elevated RhoGTPase and ROCK activity (Paszek *et al.*, 2005), with the same ROCK inhibitor, also reverted their tissue phenotype toward that resembling the nonmalignant MCF10A MECs or the T4-2 MECs incubated with an $\alpha 5$ -integrin function-blocking antibody (Figure 4A, compare fifth through seventh columns with eighth column). The T4-2 MECs treated with the ROCK inhibitor assembled smaller (Figure 4C), growth-arrested (Figure 4D, bottom row), polarized acini (Figure 4A) that were similar to those generated in the presence of a function-blocking antibody to $\alpha 5$ integrin (Figure 4A, right). In marked contrast, the T4-2 MECs generated by the IgG isotype-treated control tumors or those in which $\alpha 2$ integrin was inhibited formed larger (Figure 4C), proliferative, invasive, and disorganized colonies (Figure 4A, right). These findings suggest that the ability of FN ligation of $\alpha 5\beta 1$ integrin to promote the malignant behavior of mammary tissues, *in vitro* at least, depends on cell tension.

We next confirmed whether links between tissue tension and FN-ligated $\alpha 5\beta 1$ integrin and expression of the malignant phenotype of mammary tissues existed by manipulating ECM stiffness to increase cell tension. Consistent with an enhancement of stiffness-induced induction of cell tension, we observed that MECs ectopically expressing $\alpha 5$ integrin, as opposed to those ectopically expressing high $\alpha \nu \beta 3$ integrin, demonstrated strong directional migration (Isenberg *et al.*, 2009; Hartman *et al.*, 2016) in response to a gradient of FN stiffness, or those ectopically expressing $\alpha 2$ integrin in response to a gradient of collagen I stiffness (Supplemental Figure 3C). The impact of ECM stiffness and FN ligation of $\alpha 5\beta 1$

integrin was also illustrated by a set of invasion-monitoring studies that employed the 3D tension bioreactor with and without added FN. The 3D tension bioreactor permits the rapid stiffening of the ECM from 400 to 4000 Pa through the application of a uniaxial stretch (Cassereau *et al.*, 2015). Findings revealed that nonmalignant MCF10A control preassembled MEC colonies embedded within a nonstiffened 3D collagen–rBM remained quiescent and noninvasive for 48 h, as indicated by retention of colony circularity (Figure 4F). Upon uniaxial stretch and collagen stiffening, the nonmalignant MECs still did not invade, although they did begin to project membrane protrusions (Figure 4, E and F). MEC colonies ectopically expressing higher $\alpha 5$ integrin exhibited a similar phenotype to that demonstrated by the vector control and $\alpha 2$ integrin–overexpressing MECs when embedded within the relaxed and stretched, collagenous 3D tension bioreactor, nevertheless their protrusion activity was enhanced (Figure 4, E and F). However, and importantly, addition of FN to the 3D tension bioreactor significantly enhanced the protrusive behavior of the control mammary colonies and induced the rapid and significant invasion of the MEC colonies ectopically expressing the higher cell-surface $\alpha 5$ integrin (Figure 4E, compare with and without FN, see white arrows). These findings imply that FN ligation of $\alpha 5\beta 1$ integrin could promote the malignant behavior of mammary tissues by enhancing tumor cell tension.

FN-ligated $\alpha 5\beta 1$ integrin and not collagen-ligated $\alpha 2\beta 1$ integrin increases MEC tension

We next asked whether FN-ligated $\alpha 5\beta 1$ integrin induced more actomyosin tension in the nonmalignant MECs and whether this modified their integrin adhesions (Regent *et al.*, 2011). Collagen–FN gel contraction assays revealed that both the nonmalignant HMT-3522 and MCF10A MECs ectopically expressing higher $\alpha 5$ integrin contracted 3D collagen–FN gels better than the MECs engineered to express higher $\alpha 2$ integrin (Supplemental Figure 2). These findings were verified by traction force microscopy (TFM) which directly demonstrated that the nonmalignant MECs ectopically expressing higher $\alpha 5$ integrin exerted higher maximum cell traction force on an FN ECM, as compared with nonmalignant MECs ectopically expressing $\alpha 2$ integrin plated on collagen I ECM (Figure 5, A and B). The nonmalignant MECs expressing more $\alpha 5$ integrin that were plated on FN also showed significantly higher levels of nuclear yes-associated protein (YAP), reflecting enhanced activity of the mechano-activated Hippo pathway (Aragona *et al.*, 2013), as compared with the nonmalignant MECs expressing higher $\alpha 2$ integrin plated on collagen I (Figure 5C, quantified in Figure 5D). Indeed, the FN-ligated nonmalignant MECs expressing higher $\alpha 5\beta 1$ integrin assembled more and apparently larger peripheral integrin adhesions (Figure 5E) that stained strongly with p^{397} FAK (Figure 5E, quantified in Figure 5F). Immunostaining also showed that MECs with FN-ligated $\alpha 5\beta 1$ integrin recruited more of the force-activated molecules vinculin (Figure 5G, quantified in Figure 5H) and zyxin (Figure 5I, quantified in Figure 5J) to their ECM adhesions, as compared with MECs with collagen 1-ligated $\alpha 2\beta 1$ integrin (Figure 5, E–J). These findings show that FN ligation of $\alpha 5\beta 1$ integrin induces a greater amount of actomyosin tension in MECs at their ECM adhesions. The higher FN-ligated $\alpha 5\beta 1$ -integrin adhesion force in turn permits the assembly of tension-regulated scaffolding molecules and enhances mechano-signaling through YAP.

FN-ligated $\alpha 5\beta 1$ integrin enhances mechanotransduction in MECs

Unlike $\alpha \nu$ integrin, $\alpha 5$ integrin binds to both the proline-histidine-serine-arginine-asparagine (PHSRN) synergy and RGD sites of FN,

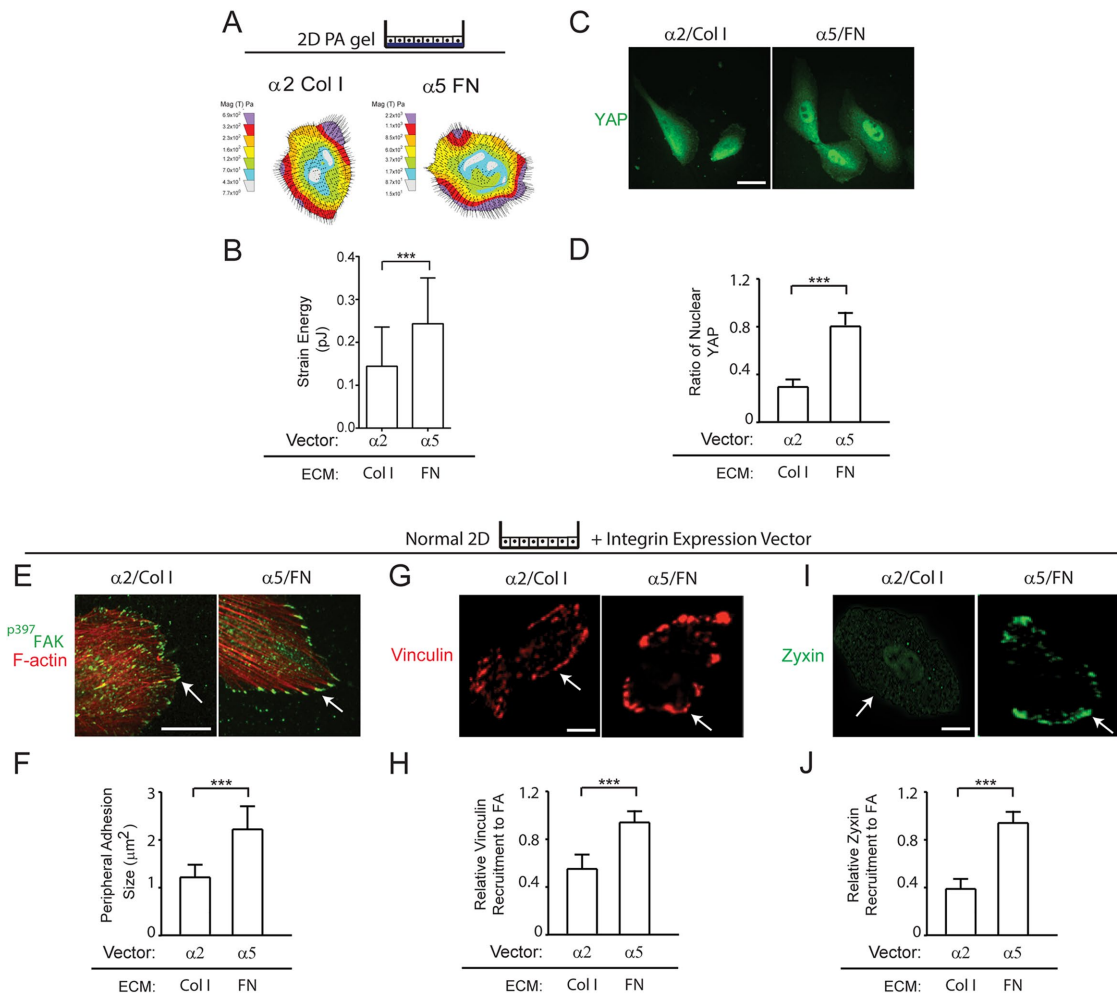


FIGURE 5: FN-ligated $\alpha 5 \beta 1$ integrin and not collagen-ligated $\alpha 2 \beta 1$ increases MEC cell tension. (A) Force maps of $\alpha 2 /$ Col I and $\alpha 5 /$ FN nonmalignant MECs and bar graphs (B) showing maximum traction generated by MECs at the cell edge. (C) Immunofluorescence images of $\alpha 2 /$ Col I and $\alpha 5 /$ FN nonmalignant MECs stained for YAP (arrow indicating nuclear localization of YAP). Scale bar: 15 μ m. (D) Bar graphs quantifying percent nuclear YAP in MECs shown in C. Results are the mean \pm SEM of three separate experiments with 10–15 acini analyzed per condition. (E) Immunofluorescence confocal images of nonmalignant MECs expressing either exogenous $\alpha 2$ integrin plated on type I collagen ($\alpha 2 /$ Col I) or exogenous $\alpha 5$ integrin plated on FN ($\alpha 5 /$ FN) stained for p³⁹⁷FAK (green) or with phalloidin (F-actin; red). Scale bar: 10 μ m. (F) Bar graph quantifying size of peripheral adhesions shown in E. (G) Immunofluorescence confocal images of $\alpha 2 /$ Col I and $\alpha 5 /$ FN nonmalignant MECs stained for vinculin (red). Scale bar: 3 μ m. (H) Bar graphs showing quantification of relative amount of vinculin recruited to focal adhesions in MECs shown in G. Quantification is relative to $\alpha 5$ -overexpressing cells on FN-coated matrices. (I) Immunofluorescence confocal images of $\alpha 2 /$ Col I and $\alpha 5 /$ FN nonmalignant MECs stained for zyxin (green). Scale bar: 3 μ m. (J) Bar graphs showing quantification of relative zyxin recruited to focal adhesions in MECs shown in I. Quantification is relative to $\alpha 5$ -overexpressing cells on FN-coated matrices. Arrows in C, E, G and I indicate peripheral localization of adhesion molecules. Results are the mean \pm SEM of three separate experiments with 10–15 cells analyzed per each condition for each experiment. ***, $p < 0.001$. Scale bars: 50 μ m.

and only FN-bound $\alpha 5 \beta 1$ integrin, but not $\alpha \nu \beta 3$ integrin, exhibits a unique adhesive bond phenotype in which ligand binding is reinforced in response to force (Friedland *et al.*, 2009; Cao *et al.*, 2012; Seong *et al.*, 2013). Consistently, shear-force adhesion studies showed that nonmalignant MECs engineered to ectopically express high levels of $\alpha 5$ integrin attached to either full-length (unpublished data) or a recombinant 9–10 domain FN, bound with much greater strength as compared with MECs plated on recombinant 9–10 domain FN in which the PHSRN domain was mutated (Figure 6A and Supplemental Figure 3B), as previously shown in fibroblasts and CHO cells (Gallant and Garcia, 2007; Friedland *et al.*, 2009). MECs

expressing higher $\alpha 5$ integrin bound with at least twice as much strength to a recombinant wild-type 9–10 domain of FN compared with MECs ectopically expressing $\alpha \nu \beta 3$ integrin ligated to the same 9–10 domain of FN. The enhanced ligand binding strength depended on synergy-site binding by $\alpha 5 \beta 1$ integrin, because the strength of adhesion decreased significantly when the synergy-site region in the 9–10 domain of FN was mutated. Given these findings and prior work emphasizing differential roles of FN domains during fibrillogenesis (Sechler *et al.*, 1997), we further explored the relevance of FN domains on cell tension generation. We first observed that the size of the integrin adhesions and the amount of p³⁹⁷FAK

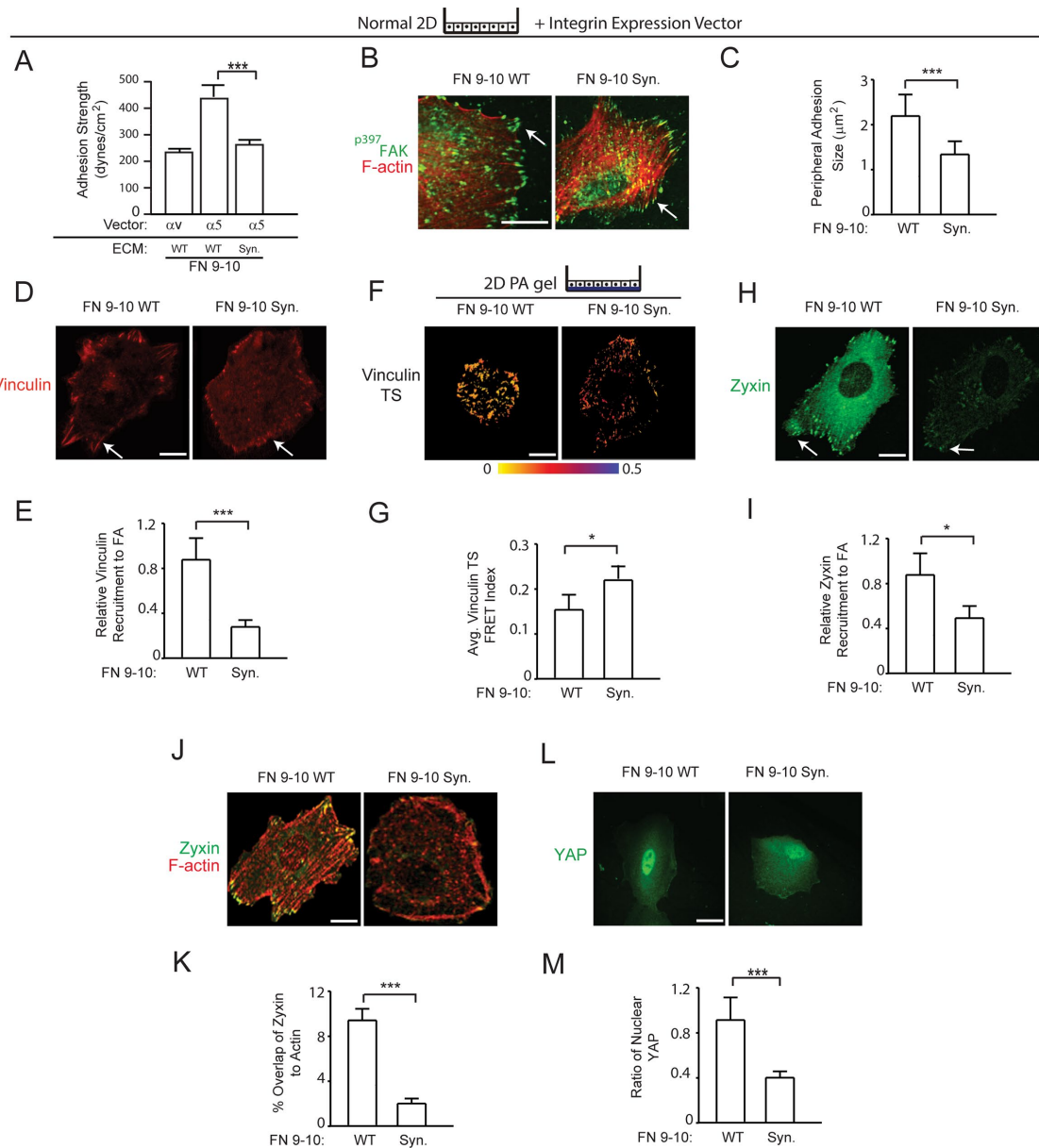


FIGURE 6: The $\alpha_5\beta_1$ -integrin adhesive bond enhances mechanotransduction in MECs. (A) Bar graphs showing adhesion strength of nonmalignant MECs expressing α_V or α_5 plated on recombinant FN 9-10 (FN 9-10 WT) and α_5 plated on recombinant FN 9-10 with the synergy-site mutated (FN 9-10 Syn). (B) Immunofluorescence confocal images of MECs expressing α_5 integrin plated on FN 9-10 WT or FN 9-10 Syn stained for p³⁹⁷FAK (green) and actin with phalloidin (F-actin; red). Scale bar: 10 μ m. (C) Bar graph quantifying size of peripheral adhesions shown in B. (D) Immunofluorescence confocal images of MECs expressing α_5 integrin plated on FN 9-10 WT or FN 9-10 Syn stained for vinculin. Scale bar: 3 μ m. (E) Bar graphs showing quantification of relative amount of vinculin recruited to focal adhesions in MECs shown in D. (F) FRET images of MECs expressing α_5 integrin and the vinculin force sensor plated on polyacrylamide gels conjugated with FN 9-10 WT or FN 9-10 Syn. Scale bar: 5 μ m. (G) Bar graphs showing quantification of FRET index at focal adhesions in MECs shown in F. (H) Immunofluorescence confocal images of MECs expressing α_5 integrin plated on FN 9-10 WT or FN 9-10 Syn stained for zyxin. Scale bar: 3 μ m. (I) Bar graphs showing quantification of zyxin recruited to focal adhesions in MECs shown in H. (J) Immunofluorescence confocal images of MECs expressing α_5 integrin plated on FN 9-10 WT or FN 9-10 Syn double-stained for zyxin and F-actin (Phalloidin). Scale bar: 3 μ m. (K) Bar graphs showing quantification of zyxin colocalization to actin in MECs shown in J. (L) Immunofluorescence images of MECs expressing α_5 integrin plated on FN 9-10 WT or FN 9-10 Syn stained for YAP. Scale bar: 15 μ m. (M) Bar graphs quantifying percent nuclear YAP in MECs shown in L. For the immunofluorescence and FRET experiments, results are represented with the mean \pm SEM of three separate experiments with either 10-15 cells (immunofluorescence analysis) or 25 cells (FRET analysis) analyzed per each condition for each experiment *, $p < 0.05$; ***, $p < 0.001$.

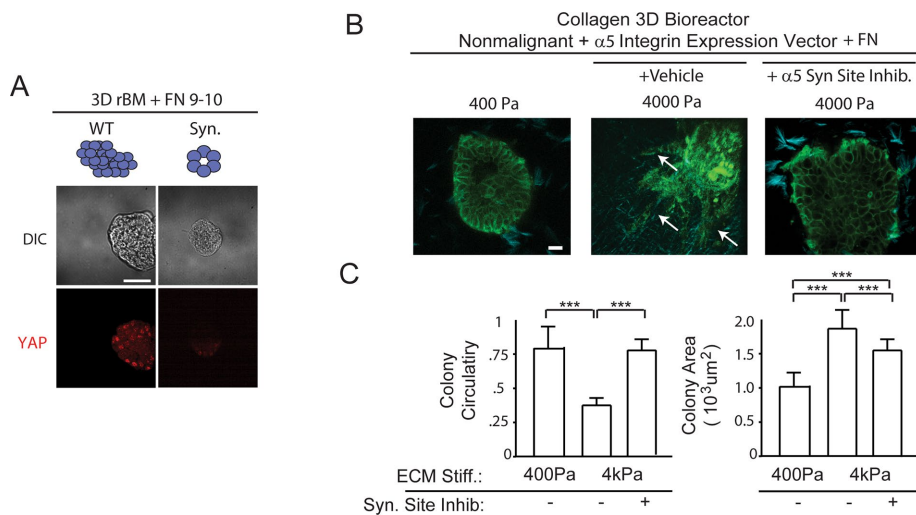


FIGURE 7: The $\alpha 5 \beta 1$ -integrin adhesive bond is required for the tension-dependent malignant behavior of MEC organoids. (A) Immunofluorescence confocal images (bottom) and phase-contrast images (top) of 3D cultures of $\alpha 5$ /FN9-10 WT and $\alpha 5$ /FN9-10 Syn. Site mutant nonmalignant MECs stained nuclear YAP. (B) Second harmonic generation images of nonmalignant (MCF10A) human mammary epithelial day 20 acini (green), expressing empty vector or elevated $\alpha 5$ integrin with FN and with or without FN synergy-site inhibitor, embedded in collagen (blue) and installed into a 3D tension bioreactor system and subjected either to 0% (400 Pa) or 10% (4000 Pa) stretch. Arrows in B indicate invasive cells, and colony circularity (left) and area (right) are quantified in C. Results are the mean \pm SEM of three separate experiments with at least 10 acini analyzed per each condition each time. ***, $p < 0.001$, one-way ANOVA. Scale bars: 50 μ m.

significantly decreased when the MECs expressing higher $\alpha 5$ integrin were plated on a recombinant 9-10 domain of FN in which the synergy site was mutated (Figure 6B, quantified in Figure 6C). Similarly, the amount of the mechano-activated molecule vinculin that appeared to be recruited to the integrin adhesions was substantially reduced when the MECs were plated on an FN lacking the synergy site (Figure 6D, quantified in Figure 6E) (Dumbauld *et al.*, 2010). Consistently, the vinculin fluorescent resonance energy transfer (FRET) index acquired using a vinculin FRET force sensor showed a significant decrease in tension applied to the vinculin molecule in the MECs plated on the 9-10 domain of FN with the mutant synergy site as compared with the wild-type domain (Figure 6F, quantified in Figure 6G) (Grashoff *et al.*, 2010). The amount of zyxin, which is also force activated (Hirata *et al.*, 2008; Hoffman *et al.*, 2012), that was recruited to actin stress fibers and localized at the integrin adhesions was similarly reduced in the MECs expressing higher $\alpha 5$ integrin when they were plated on the recombinant FN lacking the PHSRN synergy domain (Figure 6, H-K). The importance of the $\alpha 5 \beta 1$ -integrin adhesive bond in enhancing mechanosignaling in MECs was illustrated by a dramatic reduction in nuclear YAP in the MECs ectopically expressing high $\alpha 5$ integrin plated on the 9-10 domain of FN in which the synergy site was mutated (Figure 6L, quantified in Figure 6M). These findings illustrate the importance of the FN synergy site for the engagement of the $\alpha 5 \beta 1$ integrin and the enhancement of MEC tension subsequent to integrin adhesion maturation and increased cellular mechanosignaling.

$\alpha 5 \beta 1$ -Integrin engagement by the RGD and synergy site of FN is required for the tension-dependent malignant behavior of MEC organoids

We next investigated the relevance of the $\alpha 5 \beta 1$ -integrin adhesive bond in the tension-induced malignant behavior of mammary tissues in culture. MECs ectopically expressing high $\alpha 5$ integrin were

grown within rBM gels doped with either a recombinant 9-10 domain wild-type FN or recombinant 9-10 domain FN in which the synergy site was mutated and then assayed for colony morphology after 2 wk. Consistent with the importance of synergy-site ligation and activation of the $\alpha 5 \beta 1$ integrin, in the absence of the synergy site, FN-ligated MECs formed small, growth-arrested acini in rBM (unpublished data) and failed to activate the hippo pathway, as revealed by lack of nuclear YAP (Figure 7A, right). By comparison, the MECs within rBM doped with a wild-type 9-10 FN grew to form large, disorganized colonies and showed abundant nuclear YAP immunostaining (Figure 7A, left). To directly test the importance of the $\alpha 5 \beta 1$ -integrin adhesive bond in the tension-dependent malignant behavior of mammary tissues, we studied the impact of the synergy site of FN in permitting stiffness-induced MEC invasion in the 3D tension bioreactor doped with collagen-rBM-FN (see Figure 3) (Cassereau *et al.*, 2015). Mammary colonies expressing high $\alpha 5$ integrin embedded within the compliant (400 Pa) non-stretched tension bioreactor remained quiescent and noninvasive (Figure 7B, left image, quantified in Figure 7C, left and

right bar graphs). In response to uniaxial stretch and gel stiffening (4000 Pa), MECs from the colony invaded into the gel (Figure 7B, middle, see white arrows) and grew larger, as indicated by a significant drop in colony circularity (Figure 7C, left bar graph) and an increase in colony size (Figure 7C, right bar graph). However, inhibition of synergy-site binding, through treatment of the colonies with the small molecule ATN-161, prevented MEC invasion in response to ECM stiffness (Figure 7B, right, quantified in Figure 7C, left bar graph). The findings indicate that FN-ligated $\alpha 5 \beta 1$ integrin, by virtue of its unique ability to bind to the FN synergy site, enhances cell tension to induce mechanosignaling that promotes the malignant behavior of MECs.

$\alpha 5 \beta 1$ -integrin ligation by the RGD and synergy site of FN enhances MEC tension and promotes malignancy by amplifying growth factor-stimulated PI3K signaling

We next investigated how elevated cell tension mediated by ligation of $\alpha 5 \beta 1$ integrin by FN promoted the malignant behavior of MECs. EGFR signaling enhances MEC growth and survival by activating PI3K and extracellular signal-regulated kinase (ERK) (Zahir *et al.*, 2003; Yuan and Cantley, 2008), and oncogenic transformation requires PI3K and ERK signaling (Turner and Grose, 2010; Kandoth *et al.*, 2013; Stephen *et al.*, 2014). Consistently, we observed greatly reduced $p^{202/204}$ ERK and p^{473} Akt levels in the mammary epithelium of Her2/Neu mice in which collagen cross-linking and ECM stiffening had been prevented by inhibiting LOX activity (Figure 8A) (Levental *et al.*, 2009), and this reduction in PI3K and ERK signaling correlated with lower p^{397} FAK, p^{MLC} , $\alpha 5$ -integrin, and FN expression in the tissues (Figure 1B). Culture assays also showed that FN ligation of $\alpha 5 \beta 1$ integrin increased the levels and duration of EGFR-stimulated p^{473} Akt and $p^{202/204}$ ERK activity in nonmalignant and malignant MECs (Supplemental Figure 4). Thus FN-ligated, nonmalignant HMT-3522 S1 MECs expressing $\alpha 5 \beta 1$ integrin showed a 300% increase in

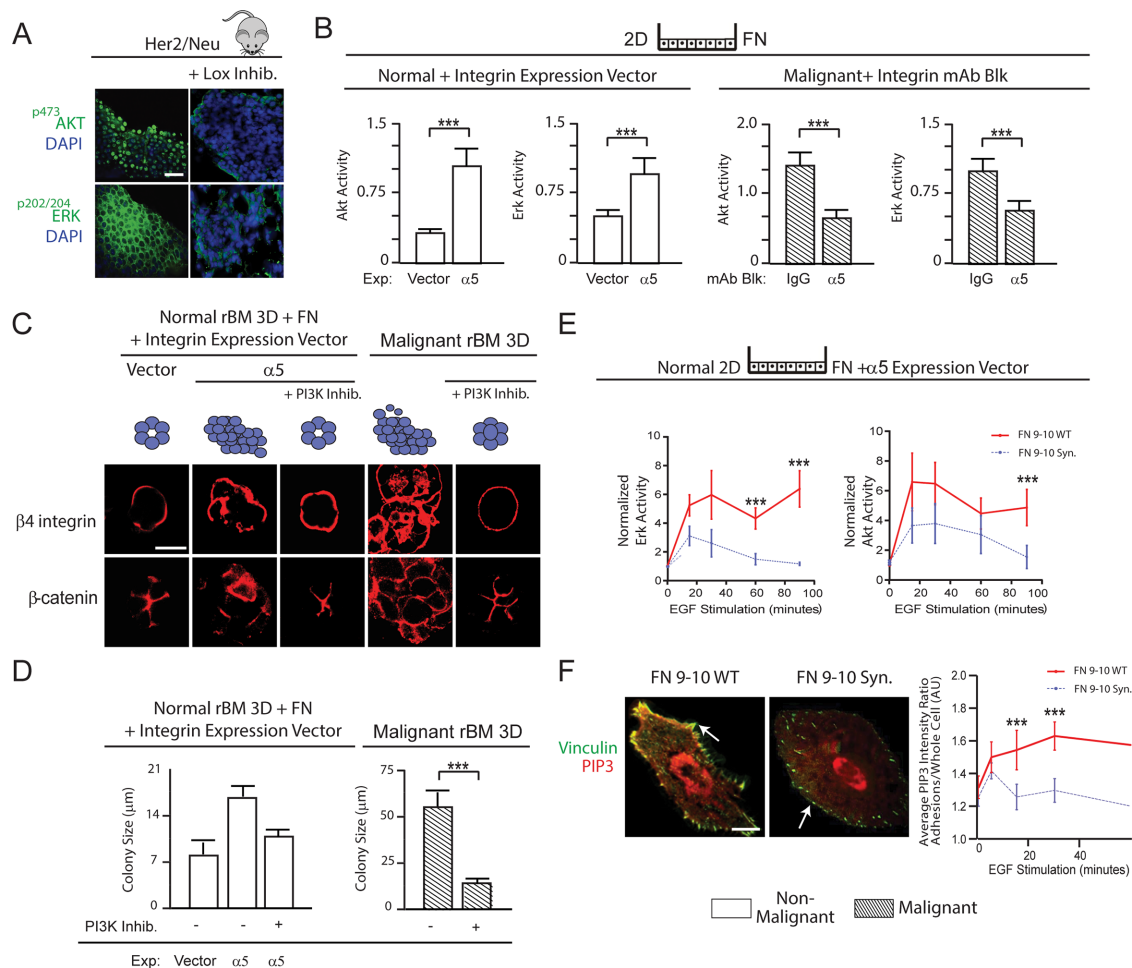


FIGURE 8: The FN synergy site–ligated $\alpha 5\beta 1$ integrin increases MEC tension and promotes malignancy by amplifying PI3K signaling. (A) Confocal immunofluorescence images of pAkt substrate, p^{Thr202/pTyr204}ERK staining, and DAPI-stained nuclei of mammary tissue excised from 7-mo-old Her2/Neu transgenic mice (three mice/condition, three to five images per mouse used for analysis) treated with or without lysyl oxidase inhibitor (Lox). Scale bar: 10 μ m. (B) Bar graphs showing level of p⁴⁷³Akt and p^{Thr202/pTyr204}ERK normalized to total cellular Akt and Erk in control or $\alpha 5$ integrin–expressing nonmalignant HMT-3522 S-1 MECs and in T4-2 malignant MECs treated with either nonspecific IgG or a function-blocking antibody to $\alpha 5$ integrin at 90 min following EGF treatment. (C) Confocal immunofluorescence images of $\beta 4$ integrin and β -catenin in colonies of control or $\alpha 5$ integrin–expressing nonmalignant HMT-3522 S-1 MECs and in T4-2 colonies treated with and without the PI3K inhibitor LY294002. Scale bar: 10 μ m. (D) Bar graphs showing size of nonmalignant and malignant MEC colonies in C. (E) Line graphs showing time course of EGF-stimulated p^{Thr202/pTyr204}ERK (top) and p⁴⁷³Akt (bottom) levels normalized to total ERK and Akt in nonmalignant MCF10A MECs expressing elevated $\alpha 5$ integrin plated on wild-type (WT) or synergy site–mutated (Syn) FN. (F) Confocal images of nonmalignant MEC cells expressing elevated $\alpha 5$ integrin, a probe for PIP3 activity (mKO2-PH-Grp1), and the focal adhesion protein vinculin plated on recombinant 9–10 FN with (WT) or without (Syn) site mutated. Scale bar: 3 μ m. Line graphs showing time course of quantification of EGF-stimulated PIP3 recruited to focal adhesions in nonmalignant MECs plated on wild-type (WT) or synergy site–mutated (Syn) FN. Measurements of all pixels in adhesions were averaged over the whole cell. Results are the mean \pm SEM of three separate experiments for all studies; at least 10 cells/condition were used for each experimental repeat or 15–20 colonies/condition for 3D culture studies. ***, $p < 0.001$.

p⁴⁷³Akt and a 200% increase in p^{202/204}ERK 90 min after EGF treatment compared with control cells expressing empty vector (Figure 8B, left bar graphs). Similarly, treating malignant HMT-3522 T4-2 MECs plated on FN with a function-blocking antibody to $\alpha 5$ integrin significantly decreased p⁴⁷³Akt and p^{202/204}ERK activation in response to EGF (Figure 8B, right bar graphs). Nonmalignant S1 MECs cultured in 3D RBM gels doped with FN formed disorganized, invasive, and nonpolarized colonies, yet inhibition of PI3K using a small-molecule inhibitor (LY294002, 5 μ M) (Figure 8C, left bar graphs) or MAP kinase inhibitor (PD98059, 20 μ M) (unpublished data) reverted their

disorganized phenotype toward differentiated nonmalignant MEC acini (Figure 8C, left bar graphs). Inhibition of either PI3K (Figure 8D, left bar graphs) or ERK activity (unpublished data) also significantly decreased the colony size of the HMT-3522 nonmalignant S1 MECs with FN-ligated $\alpha 5\beta 1$ integrin. Treatment of malignant T4-2 MECs with the same PI3K (Figure 8C, right bar graphs) or ERK inhibitor (unpublished data) also reverted the malignant phenotype to that exhibited by noninvasive, growth-arrested, polarized, nonmalignant MEC acini and significantly decreased their colony size (Figure 8D, right bar graphs). Importantly, the $\alpha 5\beta 1$ integrin–mediated increase

in PI3K signaling required ligation of the synergy site of FN, because nonmalignant $\alpha 5 \beta 1$ integrin-expressing MCF10A MECs showed a profound and sustained increase in EGF-stimulated Akt and ERK activation only when the cells were able to ligate a recombinant FN with an intact synergy site (Figure 8E).

A force-stabilized vinculin-talin-actin-zyxin scaffolding complex facilitates PI3K-mediated conversion of phosphatidylinositol (3,4)-biphosphate (PIP2) into phosphatidylinositol (3,4,5)-triphosphate (PIP3) (Rubashkin *et al.*, 2014). We therefore asked whether the force-mediated stabilization of the vinculin-talin-actin-zyxin scaffolding complex by FN ligation of $\alpha 5 \beta 1$ integrin required engagement of the FN synergy site to enrich PIP3 at integrin adhesions. Consistently, a greater amount of mKO2-PH-Grp1 (a PIP3 localization reporter; Martin-Belmonte *et al.*, 2007) could be detected at the focal adhesions (indicated by vinculin mEmerald) following EGF stimulation in the nonmalignant MCF10A MECs overexpressing the $\alpha 5$ integrin that were plated on recombinant 9–10 FN domains, but only when the synergy site was intact (Figure 8F). These findings demonstrate that the engagement of an FN-ligated $\alpha 5 \beta 1$ integrin increases cell tension to stabilize a vinculin-talin-actin-zyxin scaffolding complex that is able to promote the malignant behavior of MECs by amplifying PI3K signaling in response to EGFR stimulation.

DISCUSSION

Here we provide evidence that the FN associated with stiffened type I collagen fibers in the tumor microenvironment could foster malignant transformation and tumor progression by promoting the engagement of $\alpha 5 \beta 1$ integrin to increase tumor cell mechanosignaling. The findings are consistent with previous data supporting the role of FN in mediating the cellular response to extracellular mechanical cues (Carragher *et al.*, 2013). Our data suggest that the activation of FN-ligated $\alpha 5 \beta 1$ integrin provides the mechanical strength to transmit higher adhesion forces that promote the recruitment of vinculin, zyxin, and other focal adhesion proteins, which ultimately drive PIP nucleation at the plasma membrane to enhance growth factor-dependent PI3K signaling in the invading tumor cells. The data present a plausible explanation as to why a stiffened collagen ECM potentiates PI3K activation to promote tumor progression and provides insight into the relationship between the consistent upregulation of $\alpha 5 \beta 1$ and FN in many tumors as well as its association with metastasis. Using two-dimensional (2D) and 3D culture assays, a 3D tension bioreactor, and mouse models, we showed that FN-ligated $\alpha 5 \beta 1$ integrin, by virtue of its ability to enhance cell tension through its synergy site, can promote expression of the malignant phenotype of MECs *in vitro* and *in vivo*. Our findings provide one perspective for why $\alpha 5 \beta 1$ integrin and its ligand FN are so frequently elevated in many solid tumors, where interstitial pressure and tension are also high, and in contractile primary and metastatic cancer cell lines and tumor cells, which have high Rho and ROCK activity (Maschler *et al.*, 2005; Yao *et al.*, 2007; Craig *et al.*, 2009; Dingemans *et al.*, 2010; Nam *et al.*, 2010; Mierke *et al.*, 2011; Yu *et al.*, 2012). Our data are also consistent with prior results showing that cancer cell lines expressing abundant FN, when sorted for high membrane $\alpha 5$ -integrin levels, migrate faster and contract collagenous matrices to a greater extent (Mierke *et al.*, 2011), and studies showing that blocking the activity of RGD-binding receptors is critical for expression of the malignant phenotype of cultured breast cancer cells (Park *et al.*, 2006; Nam *et al.*, 2010). Here we not only identified $\alpha 5 \beta 1$ integrin as the key RGD receptor, but we also demonstrated that the ability of $\alpha 5 \beta 1$ integrin to promote the malignant phenotype of MECs requires binding to both the RGD and synergy site of

FN, because this permits its critical adhesive bond activation, which is required to enhance cell tension and integrin mechanosignaling (Figures 3 and 4). Importantly, prior data showed that unfolding of FN reveals the otherwise hidden synergy site that permits the engagement $\alpha 5 \beta 1$ integrin (Friedland *et al.*, 2009; Seong *et al.*, 2013). Our data expand upon these observations to imply that collagen-mediated ECM stiffening could promote malignancy by fostering $\alpha 5 \beta 1$ -integrin binding to the FN synergy site along stiff collagen fibrils that then permit the force-dependent exposure of the synergy site on FN molecules (Baneyx *et al.*, 2002; Barker *et al.*, 2005; Martino *et al.*, 2009; Cao *et al.*, 2012; Seo *et al.*, 2015).

Tumors contain abundant quantities of type I collagen, and cross-linked, remodeled type I collagen contributes critically to the tensile strength of a tissue (Levental *et al.*, 2009; Lopez *et al.*, 2011). Nevertheless, we failed to quantify differences in $\alpha 2$ integrin in either Her2/Neu mouse mammary tissue or in the 3D organotypic cultures of malignant MECs in which the tension had been reduced (Figures 1 and 2). Instead, we established a relationship among cell tension, ECM stiffness, and elevated expression of the FN receptor $\alpha 5 \beta 1$ integrin in mouse mammary tissue and mammary tumor organoids. We determined that MECs with FN-ligated $\alpha 5 \beta 1$ integrin, but not collagen I-ligated $\alpha 2 \beta 1$, exerted higher traction forces and were able to contract collagen gels better (Figure 5 and Supplemental Figure 2). Our data are consistent with prior studies suggesting that $\alpha 2 \beta 1$ integrin represses expression of the malignant phenotype of MECs in culture and is a tumor suppressor in the mammary gland *in vivo* (Zutter *et al.*, 1995) and recent data indicating that $\alpha 2 \beta 1$ integrin activates FAK by a force-independent mechanism (Seong *et al.*, 2013). Interestingly, FN and collagen are often secreted and processed in tandem (Ledger *et al.*, 1980), and an FN matrix can serve as a scaffold to guide collagen assembly (Singh *et al.*, 2010; Kubow *et al.*, 2015). Indeed, just as FN deposition and unfolding requires a collagen scaffold, collagen assembly and remodeling require FN (McDonald, 1988; Dallas *et al.*, 2005, 2006; Kadler *et al.*, 2008), during which time FN can be cross-linked to collagen (Eyre *et al.*, 1984; Mosher, 1984). FN fibril assembly and collagen remodeling form a feedback loop, with collagen-mediated ECM stiffness and cell contractility inducing conformational changes in FN that reveal hidden binding sites required for FN matrix assembly, which then further facilitate collagen remodeling (Baneyx *et al.*, 2002; Li *et al.*, 2003). Thus our data are consistent with a paradigm wherein the highly cross-linked collagen forms the scaffold upon which an FN meshwork is assembled. This stiffened ECM, by virtue of its ability to modify FN, would then favor tumor progression by permitting $\alpha 5 \beta 1$ -integrin binding to enhance tension-dependent signaling in tumors (Cao *et al.*, 2012; Seo *et al.*, 2015). Nevertheless, it is also feasible that FN-bound $\alpha 5 \beta 1$ integrin could collaborate with other transmembrane collagen receptors such as the discoidin domain receptor (DDR) or syndecans to foster malignant progression, particularly because DDRs are also tension regulated (Lieberbach and Sanderson, 1994; Shyu *et al.*, 2005; Shintani *et al.*, 2008; Vuoriluoto *et al.*, 2008).

PI3K and ERK regulate cell growth, survival, and invasion, and the levels and activity of these kinases are frequently elevated in tumors (McCubrey *et al.*, 2007; Chappell *et al.*, 2011). Accordingly, an assortment of pharmacological inhibitors have been developed to target these kinases and their associated signaling molecules to treat various solid cancers with various degrees of clinical success (McCubrey *et al.*, 2008; Engelman, 2009; Wong *et al.*, 2010; Thorpe *et al.*, 2015). Here we determined that, while the Her2/Neu tumors, which are surrounded by a stiffened ECM, have elevated ERK and PI3K activity as expected, merely preventing collagen cross-linking and stiffening by inhibiting LOX activity significantly reduced both

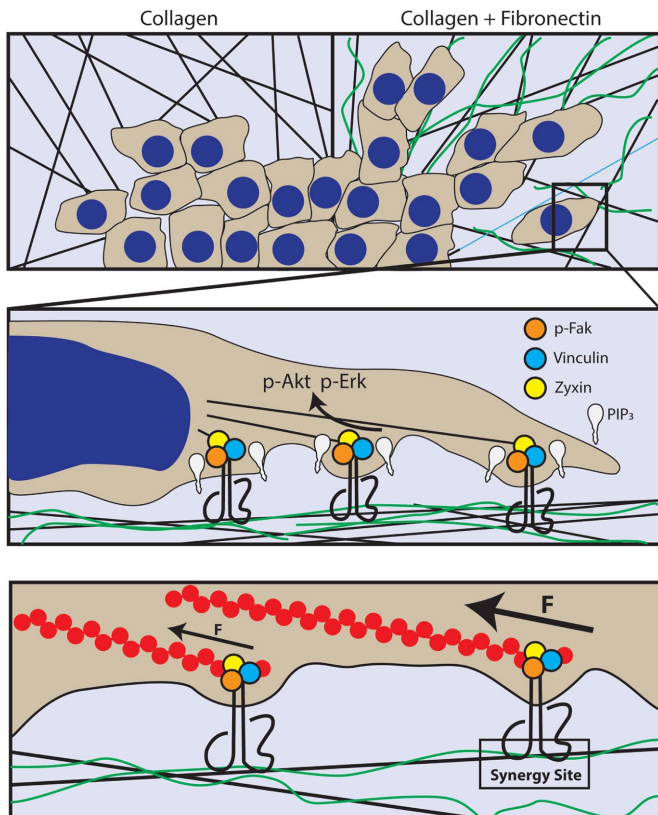


FIGURE 9: Model describing how ligation of $\alpha 5 \beta 1$ -integrin synergy site promotes MEC malignancy. When tumor cells encounter FN-rich, stiff collagen fibers at invasive fronts, they engage the $\alpha 5 \beta 1$ -integrin synergy site to recruit vinculin and zyxin to sites of focal adhesions in a tension-dependent manner to nucleate PIP₃ at the plasma membrane and enhance PI3K-dependent tumor cell invasion.

ERK and Akt activity and did so in tandem with a reduction in $\alpha 5$ integrin and FN. We also observed that MECs with $\alpha 5 \beta 1$ integrin ligated by a wild-type, but not a synergy-site mutated FN, nucleated more vinculin-talin-actin-zyxin scaffold and recruited more PIP₃ to amplify EGF-dependent activation of Akt and ERK. Inhibiting PI3K or ERK repressed the malignant behavior of FN-ligated mammary MEC 3D tissue-like structures expressing elevated $\alpha 5 \beta 1$ integrin (Figure 9). Our findings highlight the possibility that a stiffened tumor ECM stroma could amplify oncogenic signaling by enhancing EGFR signaling through PI3K and ERK via ligation of $\alpha 5 \beta 1$ integrin by the FN synergy site. The net result would be a reduced efficacy of many of the therapies targeting this pathway, as has been recently demonstrated (Hirata *et al.*, 2015). The data thereby provide one plausible explanation for why, in some instances, targeted molecular therapies are less effective and suggest that combinatorial treatments that target both the mechanical properties of the cell or tissue and specific oncogenic signaling pathways might prove to be a better therapeutic option (Sanz-Moreno *et al.*, 2011; Calvo *et al.*, 2013; Sadok *et al.*, 2015; Jiang *et al.*, 2016; Laklai *et al.*, 2016). Our findings are therefore clinically relevant, as they identify a tumor-specific mechanical signature, because the synergy site is only engaged in a high-force environment, such as in a wound or at the invasive front of a tumor. Thus inhibiting the ability of $\alpha 5 \beta 1$ integrin to ligate the synergy site of FN could prove to be a tractable cancer-specific therapy (Garcia *et al.*, 1998; Baneyx *et al.*, 2002). The data also suggest that new tools that are being developed to detect the

FN synergy site in tumor biopsies might be useful biomarkers to identify patients with potential kinase-treatment resistance (Barker *et al.*, 2005).

MATERIALS AND METHODS

Antibodies and reagents

The following primary antibodies were used for our studies: collagen IV, CIV 22, PCNA, and PC10 (DAKO); laminin-5 3-chain specific, BM165 (gift from M.P. Marinkovich, Stanford University); $\beta 1$ integrin, A1B2 (gift from C.H. Damsky, Lawrence Berkeley National Laboratory); TS2/16 (American Type Culture Collection); $\beta 4$ integrin 3E1, ASC-3, ASC-8; $\alpha 1$ integrin FB12, $\alpha 2$ integrin 10G11, $\alpha 4$ integrin P1H4, $\alpha 5$ integrin SAM-1 and P1D6, $\alpha 6$ integrin GoH3, αv integrin M9, FN, 3E3, E-cadherin, vinculin (Chemicon International); pAkt substrate, pMLCK Thr-18/Ser-19, YAP, activated caspase 3 (Cell Signaling); pMypt Thr-850 (Millipore), pFak Tyr-397 (Invitrogen); Ki-67, β -catenin, Akt and pSer-472/473/474-Akt, Erk1 (BD Transduction Laboratories); p-Erk1/2 (Thr-202/Tyr-204) (New England BioLabs); Flt-1/VEGFR1 antibody-1 (NeoMarkers). Phalloidin and anti-mouse/rabbit/rat IgG secondary antibodies, conjugated with fluorescein isothiocyanate (FITC), Texas red (Jackson Laboratories), Alexa Fluor 488/555/647 (Invitrogen), and horseradish peroxidase (HRP; Amersham Pharmacia Biotech) were used. Inhibitors used were: EGFR-specific tyrosine kinase Tyrphostin AG 1478 (200 nM), MEK1: PD98059 (20 μ M) and PI3K LY 294002 (20 μ M) (BIOMOL), FN synergy-site inhibitor (50 μ M; gift from A.P. Mazar). In addition, the following proteins were used: FN synergy-site mutants (gift from H. P. Erickson, Duke University), human recombinant VEGF Receptor 1 Flt-1 (Oncogene), and RGD peptide (Biomol International).

Cell culture

HMT-3522, MCF10A MECs, and HDMVECs were grown and manipulated in 2D and 3D rBM (Collaborative Research) and collagen matrices. Phenotypic reversion of T4-2 cells was as previously described (Weaver *et al.*, 1997). For inhibition of integrin function, the 3D multicellular structures were pre-incubated with anti- $\alpha 2$, $\alpha 5$, or αv integrin-blocking antibodies or IgG isotype-matched control monoclonal antibody (mAb; 20 g IgG/ml). When added to 3D cultures, FN concentration was 50 μ g/ml (WT and mutant FN were gifts of H. P. Erickson [Duke University] and were purified as described in *Purification of FN domains 7–10*). Colony size and morphology were measured after 10–12 d in culture. Inhibition studies were performed using ROCKi Y27632 (10 μ M), PI3Ki LY294002 (5 μ M), MAPKi PD98059 (20 μ M), EGFRi tyrphostin AG 1476 (100 nM). Adult human dermal microvascular endothelial cells (HDMVECs) were grown on collagen type I-coated flasks (Collaborative Biomedical) in EGM-2 bullet kit media (Bio-Whittaker). Angiogenesis induction by MECs was assayed by coculturing 3D rBM-generated mammary organoids in cell culture inserts (0.45- μ m pore size; Biocoat; BD Labware) with HDMVECs that had been overlaid with a 1-micron layer of acellular collagen type I (BD Pharmingen). HDMVECs invasion through the collagen overlay and network formation was assessed after 2 d by staining with toluidine blue. Anchorage-independent growth was assessed using a soft agar assay. Briefly, 20,000 cells in 1.0 ml 0.35% agarose with or without integrin-blocking antibodies, as indicated, were overlaid with 1.0 ml 0.5% agarose containing 1X growth media, and colonies larger than 40 μ m in diameter were scored positive after 21 d.

Cell and tissue staining

The 3D rBM gels, as well as tissue sections, were prepared by mixing cultures with fresh collagen following embedment and freezing in sucrose with Tissue-Tek OCT compound (Miles Laboratories),

then sectioning them in 10- to 20- μ m-thick slices for analysis. All samples were incubated with primary mAbs followed directly by either FITC-, Texas red-, or Alexa Fluor-conjugated secondary antibodies and DAPI, when indicated. Nuclei were counterstained with diaminophenyl-indole (Sigma-Aldrich). Images were compared and quantified based on fluorescence intensity signal following minimal thresholding to subtract background. For mouse studies, when mice were killed, lesions were photographed, dissected, measured, macroscopically analyzed, fixed in 4% paraformaldehyde (PFA), and paraffin embedded. H&E sections were evaluated for histopathological evidence of tumor phenotype, and tissue sections were analyzed by immunofluorescence as described.

Image acquisition and analysis

All immunofluorescence images were recorded at 20–120 \times magnification and conventional images were recorded at 40–60 \times magnification. FRET images were acquired using a 60 \times /WI 1.2 NA Plan-Apo objective. Immunofluorescence and FRET images were acquired using an Olympus IX81 epifluorescence microscope with Spot color CCD camera, Nikon TE2000-U inverted microscope, Bio-Rad MRC 1024 laser-scanning confocal microscope attached to a Nikon Diaphot 200 microscope, and a spinning-disk/ total internal reflection fluorescence (TIRF) microscope setup with Andor's iXon3 EMCCD camera with the Yokogawa CSU-X1 confocal scanner, a Nikon TIRF illuminator, and a MOSAIC module for fluorescence recovery after photobleaching (FRAP) and photoactivation on a motorized Nikon Ti-E inverted microscope base. Where immunofluorescence intensity was quantified, images were taken using identical image-acquisition parameters and were immunostained using the same primary and secondary antibody solutions. Images were imported into ImageJ (NIH), and positive signal was thresholded to secondary-only controls. The thresholded images are quantified from positive signal and presented as mean pixel density per group of samples.

The 3D tension bioreactor

PyMT organoids were cultured in rBM (Corning) for 3–5 d. The organoids were isolated by solubilizing the gels using phosphate-buffered saline (PBS)-EDTA. The isolated organoids were subsequently embedded in 2.5 mg/ml collagen I solution supplemented with FN (1 μ g/ml). The collagen gels were stretched in a tension bioreactor with 0% or 10% strain for 72 h (Cassereau *et al.*, 2015). Organoids were fixed with 4% PFA overnight for subsequent immunofluorescence staining.

Flow cytometry

Cells were isolated, blocked with 1% bovine serum albumin (BSA) in PBS for 60 min, incubated with saturating concentrations of primary mAb for 60 min, washed three times, and labeled with FITC- or phycoerythrin-conjugated goat IgG (Millipore). Stained cells were washed three times and immediately analyzed on a FACScan (BD Pharmingen). All manipulations were conducted at 4°C.

PIP3 localization analysis

Stable lines of MCF10A cells expressing tagged α 5 integrin and transiently transfected with vinculin-mEmerald and KO2-PH-Grp1 (a fluorescent PIP3 probe courtesy of Keith Mostov) were plated on recombinant FN 9–10 or recombinant FN 9–10 with the synergy-site mutants. For analysis methods used, refer to the Supplemental Experimental Procedures.

Mouse studies

Cohorts of PyMT and FVB mice were maintained in accordance with University of California IACUC guidelines under protocol AN092125.

Her2/Neu mice were treated starting at 5 mo of age with BAPN (3 mg/kg; Spectrum) in the drinking water or a LOX function-blocking polyclonal antibody (3 mg/kg; OpenBiosystems) injected intraperitoneally twice per week (Levental *et al.*, 2009). Mice were killed at 7–8 mo of age, at which time the fourth mammary gland was PFA fixed, paraffin sections were analyzed for histology, and parallel sections were stained as described.

PyMT mice were treated with BAPN (3 mg/kg; Spectrum) in the drinking water starting at 4 wk of age. The mice were killed at 14 wk of age with the tumors being harvested for PFA fixation and paraffin embedding for staining as described.

Four-week-old BALB/c nu/nu mice were subcutaneously injected in the rear flanks (5E6 cells/injection, together with Matrigel with or without addition of function-blocking antibody or IgG isotype control). Tumor development was monitored via external palpation, and lesions were measured and monitored biweekly for 2 mo upon detection (instant readout digital calipers; Electron Microscopy Sciences).

Retroviral and lentiviral infections and vectors

Standard retroviral and lentiviral infection procedures were used. For detailed descriptions of lentiviral and retroviral constructs and vectors, please refer to the Supplemental Experimental Procedures.

Western and ELISA procedures

Equal amounts of cell protein lysate (either RIPA or Laemmli lysate; BCA; Pierce) were separated on reducing SDS-PAGE gels, transferred to nitrocellulose or polyvinylidene fluoride membranes, and probed with primary antibody. Bands were visualized and quantified using a Fujifilm Gel Documentation system in combination with HRP-conjugated secondary antibodies and ECL-Plus system (Amersham Pharmacia). Specific activity for Akt and Erk was calculated by normalizing densitometric values of phosphorylated to total AKT or ERK and E-cadherin. Integrin protein levels were assessed using nonreducing SDS-PAGE gels. VEGF, IL-8, and bFGF levels in the media of 10- to 12-day 3D rBM cultures of MECs were measured using sandwich ELISA (R&D Systems), according to the manufacturer's instructions. OD measurements were performed using a Fluoroskan Ascent FL (Labsystems).

Real time-PCR analysis

Random-primed cDNA was prepared from total isolated RNA using Trizol reagent (Invitrogen), and target cDNA sequences were quantified via real-time PCR using SYBR Green I reagent (Roche) according to the manufacturer's protocol. An Eppendorf Realplex2 quantitative PCR machine was used for all studies.

Real time-PCR primers

The following primer sequences were used: 18S: forward 5'-cggctacacatccaaggaa-3', reverse 5'-gctggaattaccgcggct-3'; FN 1: forward 5'-agtgggagacctcgagaag-3, reverse 5'-gtccctcggaacatcagaaa-3', 5'-agtgggagacctcgagaag-3', reverse 5'-gtccctcggaacatcagaaa-3', VEGF: forward 5'-caggctgcacccatggcagaa-3', reverse 5'-gcatcgcatcaggggcacaca-3'; ITGB1: forward 5'-cgaggctcatggttcattgtg-3', reverse 5'-tcccatttggcattcatttt-3'; ITGA5: forward 5'-agcctcagaaggaggagac-3', reverse 5'-ggtaatgggggtgattggtg-3', ITGA2 forward 5'-tgaccaaattctgcaggaca-3', reverse 5'-ggagccaattctggtcacct-3'; ITGAV forward 5'-ccaccaagctttggtctattc-3', reverse 5'-caggcagtgcagggttta-3'.

Cell contractility, migration, and durotaxis assays

Collagen gel contraction was measured by imaging projected gel areas of cell-embedded collagen gels as described previously

(Asaga *et al.*, 1991). Traction-force microscopy studies were performed as described by Dembo and colleagues and processed using LIBTRC-2.0 software (Dembo and Wang, 1999; Reinhart-King *et al.*, 2003). For durotaxis studies, bright-field images were captured every 5 min over 12 h using gradient-stiffness polyacrylamide gel (Isenberg *et al.*, 2009). Cell migration time-course images were compiled, and cell speed, persistence, distance, and directionality were analyzed on a single-cell basis with ImageJ (NIH) and the Chemotaxis plug-in (Meijering *et al.*, 2012).

Substrate preparation

ECM-cross-linked PA gels were prepared and mechanically analyzed as described; single stiffness substrates and mechanically gradient substrates (Lakins *et al.*, 2012) with modifications made as described in Przybyla *et al.* (2016). Briefly, two droplets, each containing 12.5 μ l of a soft (100 Pa) or stiff (60,000 Pa) acrylamide/bis-acrylamide mixture, were placed adjacent to each other on a large hydrophobic coverglass (no. 1, 45 mm \times 50 mm; Fisher Scientific) and then covered with a small circular activated coverglass (no. 1, 18-mm diameter; Fisher Scientific) to merge the drops. By carefully maintaining the interface, a uniform gradient of 3.33 Pa/ μ m along the length of substrate was achieved. Regions of different rigidities were distinguished by using a fluorescently labeled bis-acrylamide in the stiff solution creating a gradient of fluorescence correlated with the mechanical gradient.

Substrate elastic modulus was measured via atomic force microscopy (Asylum Research) using the Hertz model. Briefly, a silicon beaded-tip cantilever (5- μ m diameter, 0.07 N/m, silicon nitride) was used to measure 90 \times 90 μ m elastic modulus maps down the length of the gel along the mechanical gradient while simultaneously measuring fluorescence intensity with a inverted epifluorescence microscope. We used these measurements to assess the mechanical gradient and develop an algorithm for determining the elastic modulus of the gel solely from the fluorescence intensity. This model allowed us to determine the particular surface stiffness seeded cells were adhered to when tracking cell motility.

Adhesion strength assay and generation of recombinant FN

Preparation of adhesive ligands: the previously described Promega Pinpoint vector containing the sequence for a fragment spanning the 7th to 10th type III repeat of human FN (FN7–10) was cut with *N*rul and ligated to yield an expression vector for a fragment spanning the 9th to 10th type III repeat of human FN (FN9–10). The synergy-site mutant PHSAN [FN9–10(PHSAN)] was generated using the Stratagene QuikChange Site-Directed Mutagenesis kit and primers 5'-GGGTGCCCCACTCTGCGAATTCATCACCC-3' (forward) and 5'-GGGTGATGGAATTCGCAGAGTGGGGCACCC-3' (reverse). Constructs were verified by DNA sequencing. Proteins were expressed in JM109 cells (Promega) in the presence of D-biotin and purified by affinity chromatography. Protein concentration and purity were confirmed by Western blotting and Coomassie blue staining.

Preparation of micropatterned substrates. Micropatterned substrates were generated by microcontact printing of self-assembled monolayers of alkanethiols on gold using a polydimethylsiloxane (PDMS) stamp (Sylgard 184/186 elastomer kit) with circular patterns (10- μ m-diameter circles, 75- μ m center-to-center spacing). Arrays of methyl-terminated alkanethiol [HS-(CH₂)₁₁-CH₃; Sigma-Aldrich] circles were stamped onto Au-coated glass coverslips. The remaining exposed areas were functionalized with a tri(ethylene glycol)-terminated alkanethiol [HS-(CH₂)₁₁-(CH₂CH₂O)₃-OH; ProChimia Surfaces] to generate a cell adhesive-resistant background.

Patterned substrates were coated with purified adhesive ligands (20 μ g/ml), blocked with 1% heat-denatured BSA, incubated in PBS (Ca²⁺/Mg²⁺), then seeded with cells at a density of 210 cells/mm², and incubated for 16 h at 37°C.

Cell adhesion assay description. Cell adhesion to FN-coated islands was measured using a hydrodynamic spinning-disk system. Micropatterned substrates with adherent cells were spun in PBS supplemented with 2 mM dextrose for 5 min at constant speeds. The applied shear stress (τ) is given by the formula $\tau = 0.8r(\rho\mu\omega^3)^{1/2}$, where r is the radial position and ρ , μ , and ω are the fluid density, viscosity, and rotational speed, respectively. After being spun, cells were fixed in 3.7% formaldehyde, permeabilized in 1% Triton X-100, and stained with ethidium homodimer-1 (Invitrogen). Adherent cells were counted at specific radial positions using a 10 \times objective lens in a Nikon TE300 microscope equipped with a Ludl motorized stage, Spot-RT camera, and an Image-Pro analysis system. A total of 61 fields (80–100 cells per field before spinning) were analyzed, and cell counts were normalized to the number of cell counts at the center of the disk. The fraction of adherent cells (f) as a function of shear stress τ (force/area) was then fitted to a sigmoid curve $f = f_0/[1 + \exp[b(\tau - \tau_{50})]]$, where τ_{50} is the shear stress for 50% detachment, b is the inflection slope, and f_0 is the y-intercept. τ_{50} represents the mean adhesion strength for the cell population.

Vinculin tension sensor and FRET

FRET images were collected and analyzed as previously described (Graham *et al.*, 2001). Briefly, images were background subtracted and adjusted for spectral bleedthrough. Relative FRET index was calculated by taking the ratio of pixel intensity in the FRET image (donor excitation and acceptor emission) to pixel intensity in the donor image (donor excitation and emission). Average cell FRET index was calculated for each cell in a sample of 25–30 cells. Cell averages were then used to calculate a sample average, which was then used in Student's *t* tests to evaluate whether the sampled populations had different means.

Purification of FN domains 7–10 (wild-type, double, and triple synergy-site mutants)

BL21(DE3) bacteria transformed with pET constructs expressing wild-type human FN domains 7–10 (Fn7-10) and the double (R1374A/R1379A) and triple (R1374A/P1376A/R1379A) synergy-site mutants were generous gifts of H. P. Erickson (Duke University). Bacteria were grown to an OD₆₀₀ of 0.5–0.6 in 125 ml of 2 \times YT with 50 μ g/ml ampicillin at 37°C in a shaking incubator, and expression was induced for 2–3 h by addition of IPTG to 0.4 mM. Bacteria were pelleted and resuspended on ice in 5 ml of 0.2 μ g/ml lysozyme in 50 mM Tris-HCl/100 mM NaCl/1 mM EDTA, pH 8 (at 4°C). MgCl₂, DNaseI, and PMSF were added to final concentrations of 10 mM, 190 U/ml, and 1 mM, respectively, and bacteria were digested for 30 min with stirring on ice. Lysis was effected by addition of Triton X-100 0.1% and sonication with two 30-s bursts. The supernatant containing soluble Fn7–10 was obtained by centrifugation for 30 min at 15,000 \times *g* (at 4°C) and precipitated by addition of solid ammonium sulfate to 40% saturation (at 4°C). Following centrifugation for 30 min at 15,000 \times *g*, the pelleted fraction was redissolved in 10 ml of 20 mM Tris-HCl, pH 7.9 (at 4°C). Redissolved proteins were filtered (0.22- μ m pore size) and loaded at 1 ml/min onto a 1.6 cm \times 10 cm Q-Sepharose HP (GE Healthcare) column equilibrated in 20 mM Tris-HCl, pH 7.9 (at 4°C) and washed in the same until OD₂₆₀ returned to baseline. Bound proteins were eluted by a linear gradient of 0–0.5 M NaCl over 100 min, and 5-ml fractions were collected. Fractions

containing wild-type and mutant forms of Fn7–10 were identified by SDS–PAGE and staining with Coomassie blue and eluted between 0.26 and 0.35 M NaCl. Peak fractions were pooled and desalted by exhaustive dialysis against PBS and sterilized by filtration through 0.22- μ m syringe filters. Purified Fn7–10 concentrations were estimated by micro-BCA Assay (Pierce Chemical).

Doxycycline-inducible expression of α integrins

For HMT3522 cell lines S1 and T4-2, $\alpha 5$ and $\alpha 2$ integrins were cloned and expressed as doxycycline-inducible eGFP fusions in the retroviral vector pHRS puro Tet MCS. pHRS puro Tet MCS features in reverse orientation, an expression cassette for puromycin acetyltransferase back to back with a hepatamerized TetO minimal cytomegalovirus (CMV) promoter downstream of which α -integrin fusions were cloned. After transduction and selection for puromycin at 1 μ g/ml, resistant cells were transduced with the recombinant retrovirus generated from MFG tTA, which constitutively expresses the TETOFF transcriptional transactivator from the 5'LTR. Transduced cells were induced by withdrawal of doxycycline for 2 d and sorted by flow cytometry for eGFP-positive cells to obtain a more homogeneous population of $\alpha 5$ and $\alpha 2$ integrin-overexpressing cells. For MCF10A cells, overexpressing lines were prepared using recombinant lentiviruses. Expression of the synthetic reverse tetracycline transactivator (TETON) rTA^S-M2 was obtained by transduction of target cells with the lentiviral vector pLV rTA^S-M2 IRES Neo^r. In pLV rTA^S-M2 IRES Neo^r, rTA^S-M2 is under control of the human EEF1 α promoter and is coexpressed with neomycin phosphotransferase via a bicistronic (IRES-mediated) mRNA. Transduced cells were selected with 250 μ g/ml G418. The complete coding regions (cds) of human $\alpha 5$ and $\alpha 2$ integrins were subcloned into the lentiviral vector pLV puro Tet MCS. PCR was used to introduce a Kozak consensus sequence to the 5' ends of the cds and at the 3' end to remove the stop codon and enable in-frame fusion to five tandem repeats of the myc epitope recognized by mAb 9E10 followed in frame by TagRFPT fluorescence protein. α -Integrin fusions were cloned downstream of the hepatamerized TetO minimal CMV promoter in pLV puro Tet MCS. pLV puro Tet MCS expresses puromycin acetyltransferase from an internal expression cassette in reverse orientation, and transduced G418^r rTA^S-M2-expressing cells were selected with puromycin at 1 μ g/ml. Following selection, expression of integrins was obtained by induction with doxycycline and a more homogeneous expressing population was obtained by flow sorting for TagFPT-positive cells.

Statistical analysis

Statistical analysis was performed using Prism/GraphPad Software (La Jolla, CA) at indicated *p* values. Unless otherwise stated, two-tailed Student's *t* tests were used for significance testing to compare mean differences between two conditions, and one-way analysis of variance (ANOVA) was used to compare means of more than two conditions. Means were presented as \pm SEM of at least three independent experiments. Where indicated, sample size, *n*, refers to either the number of animals (for animal studies with indicated number of images analyzed per animal, and unless otherwise indicated, three to five images were analyzed per animal) or the number of times each experiment was repeated (for all in vitro studies, at least 10 cells per condition were analyzed for each replica). Statistical significance was considered at *p* < 0.05 and was calculated only for experiments with a sample size of at least three. Adhesion-strength values were specifically analyzed via ANOVA and Tukey's post hoc test with a *p* value < 0.05 considered significant.

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